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RCE of Application No. 10/060,759
Office Action dated January 17, 2006
Advisory Action dated August 23, 2006
Amendment mailed December 18, 2006

REMARKS

In the Advisory Action dated August 23, 2006, the Examiner withdrew the objection to claim 15 and indicated that claim 15 was allowable. Applicant appreciates this.

Claims 1-7 were rejected under 35 U.S.C. §103(a) as being unpatentable over Vassallo et al., Mayo Clinic Proceedings ("Vassallo").

The Examiner contends that the claimed invention contains "mere recognition of latent properties in the prior art". Applicant respectfully submits that this rejection should be withdrawn for the following reasons.

The Examiner contends that Vassallo teaches administration of theophylline. As the Examiner acknowledges, theophylline is a PDE inhibitor. In contrast, claims 1-7 are **not directed** to the a methods of using **general PDE inhibitors**. Claims 1-7 **are directed** to methods of using **specific PDE4 inhibitors** to treat a specific disease, namely chronic lymphocytic leukemia (CLL).

There are at least 11 different families of PDEs encoding at least 21 genes resulting in at least 50 different PDE gene products. In addition, theophylline also has activities that are not related to being a PDE inhibitor. Accordingly, the skilled artisan reading Vassallo would not know that one could obtain the described activity using a specific PDE4 inhibitor. Additionally, applicant has already shown that the PDE4 inhibitors, rolipram and (4-(3-Butoxy-4-mehtoxybenzyl) – 2 – imidazolidinene (XX5) are surprisingly better than theophylline (see Figures 12A, 12B and 14). Applicant is submitting a manuscript co-authroed by applicant showing that two additional PDE4 inhibitors work about as effectively as rolipram, and hence, better than theophylline (Meyers et al. entitled "PDE4 inhibitors augment levels of glucocorticoid receptor B cell chronic lymphocytic leukemia but not in normal circulating hematopoietic cells" attached herewith as Exhibit B).

More specifically, in light of the knowledge in the art relating to the vast number of different PDEs and their importance in the function of normal hematopoietic cells, a discovery that one can treat a disease, such as CLL with a **specific** PDE inhibitor allows one to achieve an

effective treatment with far less potential for **serious side effects**. These facts are discussed below in further detail.

Since the late 1970's it has been known that there are a number of different PDE families (Introduction, col. 1, p. 21, of the review article by Lerner and Epstein, Biochem J., 2006, 393: 21-41 attached herewith as Exhibit A). For example, it is currently known that there are at least 11 different PDE families encoding at least 21 genes (see, e.g., Table 1, in Exhibit A). It is also well known that PDEs control a variety of cellular processes and that they play an important role in the function of normal hematopoietic cells (p. 22-27, Exhibit A).

In treating diseases such as CLL, one must not only consider the efficiency of the treatment but also the associated side effects. As discussed in the specification at page 6, lines 20-21, there are at least three PDEs, types 1, 3 and 4, that are specifically expressed in T lyphocytes. Using combinations of the three inhibitors can cause serious side effect (col. 2, page 24, in Exhibit A, and references cited therein).

Applicant has previously taught that **theophylline is not a specific PDE4 inhibitor.** It is not even a specific PDE inhibitor. Indeed, Vassallo specifically teaches that the **mode of action** of theophylline is **poorly defined** (p. 346, 2nd col.). Theophylline is described to **have a number of different actions** at cellular level, including generally inhibiting phophodiesterases (PDEs) (p. 346, abstract, and under subtitle "Phosphodiesterase Enzyme Inhibition" bridging pp. 346 and 347). Moreover, Vassallo also states that the "**mechanism by which theophylline** induces apoptosis **is unclear**" (p. 351, 1st col., second to last sentence). Accordingly, Vassallo does not teach or suggest one could use a specific POE4 inhibitor or the specific advantages including its efficiency and reduction of harmful side effects.

The Examiner stated in the July 25, 2005 Office Action (p. 3) that **in the absence of evidence to the contrary**, it would have been obvious to treat a patient with CLL using theophylline, a non-specific PDE inhibitor. Applicants have provided Exhibits A and B to show that 1) a use of an agent that potentially has general PDE inhibitory activity would not allow one to conclude that any specific PDE inhibitor would be useful in the method of treatment of claims 1-7; and 2) the superior performance of using specific PDE4 inhibitors in inhibiting apoptosis in CLL

cells. The evidence provided therein, and **within the four corners of the specification** clearly shows that **a specific PDE4** inhibitor has a superior effects over theophylline in treating CLL. As discussed previously in the Amendment dated October 25, 2005, for example, in Example 3, Applicant clearly showed that PDE4, not PDE1, induced apoptosis in CLL cells (see, e.g., page 17 of the specification).

Moreover, as already shown in the Examples, the unexpectedly superior effect with using a specific PDE 4 inhibitor when compared to theophylline is supported in Exhibit B.

Accordingly, Applicant respectfully submits that Vassallo et al. does not render the present claims directed to using a specific PDE 4 inhibitor for the treatment of CLL obvious. Vassallo does not suggest the use of a PDE4 inhibitor any more than a PDE1 inhibitor, or a PDE VII inhibitor, etc. Vassallo does not suggest that any PDE inhibitor would be effective against CLL. Accordingly, this rejection should be withdrawn.

In view of the foregoing, Applicant respectfully submits that all claims are in condition for allowance. Early and favorable action is requested.

In the event that any additional fees are required, the PTO is authorized to charge our Deposit Account No. 19-2380.

Respectfully submitted,

Date: December 18, 2006

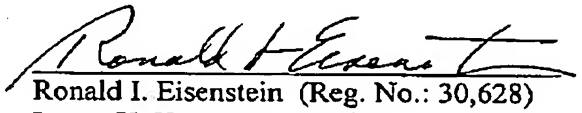

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EXHIBIT A

REVIEW ARTICLE

Cyclic nucleotide phosphodiesterases as targets for treatment of haematological malignancies

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The cAMP signalling pathway has emerged as a key regulator of haematopoietic cell proliferation, differentiation and apoptosis. In parallel, general understanding of the biology of cyclic nucleotide PDEs (phosphodiesterases) has advanced considerably, revealing the remarkable complexity of this enzyme system that regulates the amplitude, kinetics and location of intracellular cAMP-mediated signalling. The development of therapeutic inhibitors of specific PDE gene families has resulted in a growing appreciation of the potential therapeutic application of PDE inhibitors to the treatment of immune-mediated illnesses and haematopoietic malignancies. This review summarizes the expression and function of PDEs in normal haematopoietic cells and the evidence that family-specific inhibitors will be therapeutically useful in myeloid and lymphoid malignancies.

Key words: cAMP, cancer therapy, glucocorticoid, leukaemia, methylxanthine, phosphodiesterase.

INTRODUCTION

Following the identification of cAMP in 1958 by Rall and Sutherland [1], research focused for more than a decade on elucidating the role that this 'second messenger' played in regulating metabolic pathways, as well as identifying the enzymes responsible for cAMP synthesis and catabolism [1–3]. By the 1970s, however, cAMP was implicated as a regulator of cell growth (reviewed in [4–6]), and several investigators reported that elevation of cAMP levels induced arrest of proliferation or cell death in susceptible normal or malignant lymphoid populations [7–10]. Upon identifying cAMP as a second messenger, Rall and Sutherland [1] also reported the presence in tissue extracts of a caffeine (1,3,7-trimethylxanthine)-sensitive enzymatic activity, cyclic nucleotide PDE (phosphodiesterase), capable of hydrolysing cAMP. It became apparent in the 1970s that multiple forms of PDE existed [11,12] and that different forms could be inhibited differentially by pharmacological agents [12–15]. Reports in the 1970s also demonstrated that methylxanthines suppressed lymphocyte activation and proliferation [16–18] and that PDE activity in leukaemic cells was as much as 10–20-fold higher than that in normal quiescent lymphocytes [19,20]. From these observations, it was proposed, 25–30 years ago, that PDEs may be potential therapeutic targets in the treatment of haematological malignancies [12,19–21].

It is now well accepted that PDEs control a myriad of cellular processes through their ability to hydrolyse and thus control the levels of the second messenger signalling molecules, cAMP and cGMP [22,23] (Figure 1). In addition to controlling the steady-state levels of cyclic nucleotides, it has become clear that PDEs also control the spatial and temporal components of cAMP and cGMP signalling [24–26]. PDEs are encoded by at least 21 different genes, grouped into 11 different gene families, based on sequence similarity, mode of regulation and preference for cAMP or cGMP as substrate [27,28]. These 11 PDE gene families and some of their properties are presented in Table 1. With the existence of multiple transcription-initiation sites, as well as alternatively spliced forms of many of these genes, more than 50 different forms of PDE have been identified and cloned to date, many of which vary with respect to tissue distribution and the intracellular signalling pathways with which they interact. A considerable number of reviews both on PDEs in general, as well as on roles for PDEs in controlling specific cellular functions, have been written in recent years, including potential roles for PDEs as targets for treating inflammatory diseases [29–32] and cancer [21,33–35]. The present review will examine the current evidence that cyclic nucleotide PDE inhibitors will prove to be beneficial as therapeutic agents in the treatment of lymphoid and myeloid malignancies.

Abbreviations used: AKAP, A-kinase anchoring protein; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; APL, acute promyelocytic leukaemia; ATRA, all-*trans*-retinoic acid; BAD, Bcl-2/Bcl-X_L-antagonist, causing cell death; Bcl-2, B-cell lymphoma 2 anti-apoptotic protein; CLL, chronic lymphocytic leukaemia; COPD, chronic obstructive pulmonary disease; 6-CPT-2Me-cAMP, 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate; CRE, cAMP-response element; CREB, CRE-binding protein; DLBCL, diffuse large B-cell lymphoma; EAE, experimental autoimmune encephalomyelitis; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; EPAC, exchange protein activated by cAMP; ERK, extracellular-signal-regulated protein kinase; GAF, a conserved domain found in mammalian cGMP-binding PDEs, *Anabena* adenylate cyclases and *Escherichia coli* FhlA; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; H-PBL, human peripheral blood lymphocyte; IBMX, 3-isobutyl-1-methylxanthine; IκB, inhibitor of nuclear factor κB; IL, interleukin; LPS, lipopolysaccharide; 8-MM-IBMX, 8-methoxymethyl-isobutylmethylxanthine; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor κB; PAS, Per-Arnt-Sim domain; PDE, cyclic nucleotide phosphodiesterase; PHA, phytohaemagglutinin; PI3K, phosphoinositide 3-kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PML, promyelocytic leukaemia; PP2A, protein phosphatase 2A; RAR, retinoic acid receptor; RNAi, RNA interference; Rp-8-Br-cAMPS, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; SCID, severe combined immunodeficient; TCR, T-cell receptor; TNF, tumour necrosis factor; UCR, upstream conserved region.

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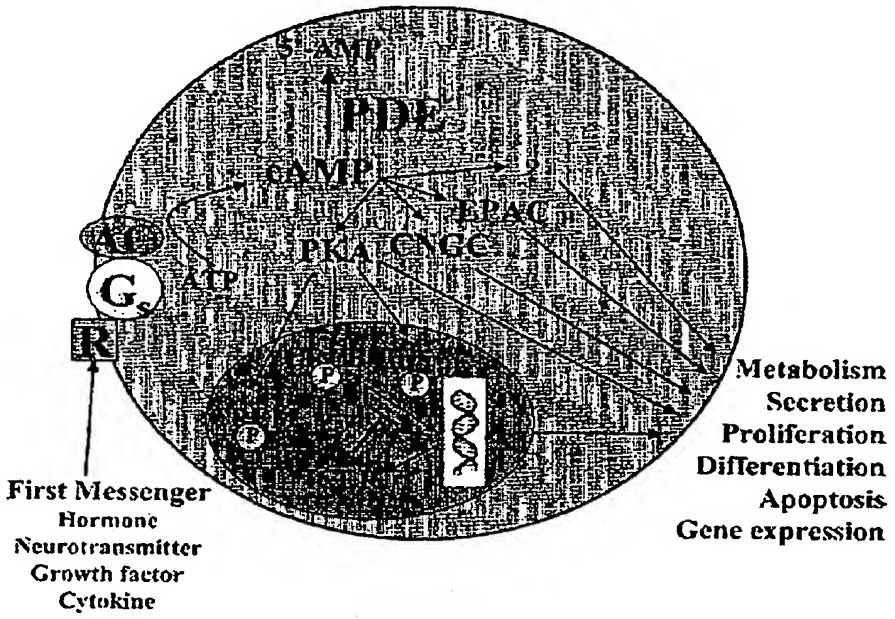

Figure 1 Role of PDEs in regulation of signal transduction

In the model of the second-messenger concept originally put forth by Sutherland and colleagues [295], first messengers, such as hormones, neurotransmitters, cytokines and growth factors, upon interacting with receptors on the cell surface, generate the production of a 'second messenger' such as cAMP, which then redirects the machinery of the cell, affecting many physiological processes. Currently, three different types of effector proteins to which cAMP can bind and carry out its actions are known: PKA [296], EPAC [251,252] and CNGCs (cyclic nucleotide-gated channels) [297]. In addition to these three known effector pathways for cAMP action, it remains possible that other currently undescribed cAMP effectors exist as well. Investigators examining cAMP inhibition of IL-5 production by T-cells, suppression of neutrophil apoptosis and inhibition of PKB/Akt leading to apoptosis in DLBCL cell lines have reported that these cAMP-mediated phenomena occur independently of currently described cAMP effector proteins [125,213,298]. cAMP effector molecules can also affect cellular processes directly through tethering mechanisms, as has been shown for the PKA catalytic subunit, which forms complexes with the GR, IκB and NF-κB [258,299]. Tethering of the PKA catalytic subunit in a complex with IκB and NF-κB provides a novel cAMP-independent means of regulating PKA, whereby degradation of IκB disinhibits PKA, allowing it to phosphorylate and activate NF-κB [299]. PKA is uniformly expressed and EPAC is expressed in specific subsets of haematopoietic cells, and these two effectors are probably responsible for mediating almost all of the known cAMP signalling events in these cells. Although CNGCs have not been explored in haematopoietic cells, they are expressed in spleen and thymus [300], and future investigations may uncover a role(s) for these effectors in these cells as well. Activation of PKA by cAMP leads to changes both in cytosolic proteins and in gene transcription through phosphorylation of cAMP-responsive nuclear factors such as CREB, CREM (CRE modulator) and ATF-1 (activating transcription factor 1) [301]. Additionally, a powerful repressor, ICER (inducible cAMP early repressor), can be formed from the CREM gene, following stimulation of cAMP signalling, probably as a feedback mechanism to terminate cAMP-induced gene expression [301]. ICER has been shown to function in T-cells as a transcriptional repressor and to be induced by the PDE4 inhibitor, rolipram, in osteoblasts [302,303]. PDEs, by regulating cAMP levels, play a central role in modulating all of these cAMP signalling pathways and consequent physiological responses. AC, adenylate cyclase; R, receptor (seven-membrane-spanning G-protein-coupled metabotropic receptor).

PDE FAMILY FUNCTION AND EXPRESSION IN NORMAL HAEMATOPOIETIC CELLS

In this section, we will review the expression patterns of PDE families within the haematopoietic system, the signalling pathways known to be regulated by these enzymes within haematopoietic cells and the availability of specific inhibitors either experimentally or clinically. Although characterization of the PDEs within normal haematopoietic cells may be at least partially predictive of their expression within corresponding haematological malignancies, an equally important aspect of this literature is that it may allow us to predict the likely haematological toxicity of PDE inhibitor therapy.

PDE1

PDE1 enzymes are widely expressed calcium- and calmodulin-dependent PDEs that catabolize both cAMP and cGMP [36–38]. The PDE1 gene family consists of three genes: PDE1A, PDE1B and PDE1C. All three of these PDE1 gene products exhibit a relatively high affinity for cGMP ($K_m \approx 1$–$5\ \mu M$), but differ in their affinities for cAMP, with PDE1C exhibiting the highest affinity ($K_m \approx 1\ \mu M$), PDE1B having an intermediate affinity ($K_m \approx 7$–$24\ \mu M$), and PDE1A showing the lowest affinity ($K_m \approx 50$–$100\ \mu M$). As a result of PDE1 calmodulin dependence, expression of this family of enzymes allows calcium signalling pathways to regulate cyclic nucleotide-dependent signalling. Although several inhibitors showing some selectivity for PDE1 have been available for a while, none are highly potent, nor truly selective for inhibition of PDE1. Vinpocetine [39] and 8-MM-IBMX (8-methoxymethyl-isobutylmethylxanthine) [40], the most commonly used PDE1 inhibitors, were originally reported to be selective for inhibition of PDE1, but subsequent studies showed 8-MM-IBMX to not be as selective as originally reported [41], and recent studies have shown that vinpocetine can inhibit other PDEs, particularly PDE4B, in the same concentration range ($IC_{50} = 22\ \mu M$) as that for the PDE1 enzymes ($IC_{50} = 5$–$25\ \mu M$) (Vince Florio, personal communication). Complicating further the use of vinpocetine for examination of cellular PDE1 effects were reports that PDE1C isolated from insulinoma cells is resistant to vinpocetine inhibition [42] and that sodium channel activation can be inhibited by vinpocetine independently of its effects on PDE [43]. The dual PDE1/PDE5 inhibitors, SCH51866 and zaprinast (M&B 22938) have sometimes been used to explore functions of PDE1, especially in tissues where PDE5

Table 1 PDE gene families

The Table shows the eleven PDE gene families, the known genes within them, their affinity constants for cAMP and cGMP, and commonly used pharmacological inhibitors for each of the families. The substrate affinity constants listed are approximate, and, where given, ranges represent the different K_m values reported for these family members in the references cited.

Family	Type	Genes	K_m cAMP (μM)	K_m cGMP (μM)	Commonly used inhibitors*	References
PDE1	CaM-dependent	1A	50–100	5	Vinpocetine (5–25)	[36–38,272–276]
		1B	7–24	3	8-MM-IBMX (4)	
		1C	1	1		
PDE2	cGMP-stimulated	2A	30	10	EHNA (1)	[59,68,69,277]
PDE3	cGMP-inhibited	3A/B	0.2–0.5	0.02–0.2	Cilostamide (0.005)	[80,278,279]
					Milrinone (0.3)	
PDE4	cAMP-specific	4A–D	1–4	–	Rolipram (1)	[130,280–283]
					RO 20-1724 (2)	
					Piclamilast (0.001)	
PDE5	cGMP-specific	5A	–	1–5	Sildenafil (0.003)	[240,241,284–287]
					Zaprinast (0.3)	
					Dipyridamole (0.9)	
PDE6	Photoreceptor	6A–C	–	20	Zaprinast (0.15)	[288,289]
					Dipyridamole (0.4)	
PDE7	cAMP-specific	7A/B	0.03–0.2	–	Dipyridamole (42, 7A; 0.5–9, 7B)	[150–152,290–292]
PDE8	cAMP-specific	8A/B	0.04–0.15	–	Dipyridamole (4–9, 8A; 23–40, 8B)	[162–164,166]
PDE9	cGMP-specific	9A	–	0.07–0.39	Zaprinast (30)	[170,171,293]
					SCH 51866 (2)	
PDE10	Dual substrate	10A	0.05–0.26	3–9	Papaverine (0.03)	[173,174,179,294]
PDE11	Dual substrate	11A	1–6	0.5–4	Tadalafil (0.07)	[183–185,187,188]
					Zaprinast (11–33)	
					Dipyridamole (0.3–1.8)	

*The numbers in parentheses are the approximate reported K_i or IC_{50} values for inhibition of that PDE gene family, given in μM. Note: the non-specific methylxanthine inhibitor IBMX inhibits all of the PDE families with the notable exception of the PDE8 and PDE9 gene families, which appear to be resistant to IBMX inhibition.

is not expressed [44,45]. The discovery that phenothiazine antipsychotics [46], as well as several other classes of pharmacological agents [47], bind to calmodulin and antagonize its stimulation of PDE1, led to the wide use of these agents, especially trifluoperazine (e.g. [48]), to probe a PDE1-controlled function. However, given the wide range of cell functions that are controlled by calmodulin, it is difficult to conclude that a process inhibited by these agents is necessarily controlled by PDE1. Another approach that has been taken is a gene-regulation approach to selectively block PDE1 expression [49]. In general, where selective inhibitors of a given PDE form are not available to probe for a physiological function of that PDE, the approach of selectively inhibiting the expression of that PDE isoform by use of oligodeoxynucleotide antisense [50] or RNAi (RNA interference) [51] techniques should prove quite useful. Recently, for example, Lynch et al. [52] used RNAi techniques in HEK-293 (human embryonic kidney) cells to demonstrate that knockdown of PDE4D5, but not other expressed PDE4 isoforms, amplifies isoproterenol-stimulated phosphorylation of the β_2-adrenergic receptor by PKA (cAMP-dependent protein kinase) and G_i-mediated activation of ERK (extracellular-signal-regulated kinase). Clearly, though, there is still a need for better, more highly selective PDE1 inhibitors for exploration of the physiological functions of this family of enzymes.

Although the majority of studies on this family of enzymes focus on their role in the cardiovascular system and brain, Jiang et al. [49] detected PDE1B1 by RT (reverse transcriptase)-PCR in activated HPBLs (human peripheral blood lymphocytes). Both transcript and enzymatic activity are induced following stimulation of HPBLs or T-cells with the T-cell mitogen PHA (phytohaemagglutinin) or CD3/CD28 respectively [49,53]. Inhibition of PDE1 in CD3/CD28-stimulated T-cells with 8-MM-IBMX or vinpocetine partially blocked IL (interleukin)-13 production [53]. PDE1 activity was not detected in resting human $CD19^+$ peripheral blood B-cells [54], but was in B-lymphoblastoid cell lines [55]. PDE1 enzymatic activity is present at very low levels in human peripheral blood monocytes and increases markedly during differentiation of monocytes to dendritic cells [56]. The splice isoform PDE1B2 is selectively up-regulated following differentiation of monocytes to macrophages because of the use of a different promoter [57]. Of note, mice rendered deficient for PDE1B by homologous recombination have exaggerated locomoter activity, but no gross haematopoietic defects were reported [58].

PDE2

PDE2 hydrolyses both cAMP and cGMP with non-linear kinetics displaying strong positive homotropic co-operativity. Half-maximal velocities for cAMP and cGMP hydrolysis are at approx. 30 μM and 10 μM respectively [59]. Additionally, PDE2 possesses two high-affinity non-catalytic cGMP-binding domains near its N-terminal end, termed GAF (a conserved domain found in mammalian cGMP-binding PDEs, *Anabena* adenylate cyclases and *Escherichia coli* FhlA) domains, to which cGMP binds with a K_d of approx. 0.3 μM and stimulates the enzymatic hydrolysis of PDE2 for cAMP [60–62]. The stimulation of PDE2 cAMP hydrolysis by cGMP is not a result of a change in V_{max}, rather, the binding of cGMP to one of the GAF domains allosterically alters PDE2 resulting in less co-operative behaviour, thus producing a lowering of the apparent K_m for cAMP. This reduction in co-operativity upon cGMP binding provides a means for small increases in cGMP to dramatically stimulate the hydrolysis of cAMP by PDE2. Indeed, as an example of this, it was demonstrated recently that ANP (atrial natriuretic peptide) stimulation of particulate guanylate cyclase in intact bovine zona glomerulosa cells led to the decrease of cellular cAMP from high micromolar levels to sub-micromolar levels in 15–20 s through cGMP

stimulation of cAMP hydrolysis by PDE2 [26]. GAF domains are also present in PDEs 5, 6, 10 and 11, although it has not been demonstrated that cGMP binding to these domains on PDEs other than PDE2 leads to activation of the catalytic activity, and the functional consequences of cGMP binding to these other PDE forms is still not entirely clear. In the case of PDE2, however, since cGMP binding to one of the GAF domains clearly activates the enzymatic hydrolysis of cAMP, expression of PDE2 allows stimuli that elevate cGMP levels to reduce cAMP signalling [63]. So far, only a single gene for PDE2, PDE2A, has been identified [64]; however, multiple alternatively spliced isoforms of PDE2A have been reported, some of which are expressed in the same cell [65–67]. Until recently, the only known agent shown to selectively inhibit PDE2 relative to other PDE isoforms was the adenosine deaminase inhibitor, EHNA [erythro-9-(2-hydroxy-3-nonyl)adenine] [68,69]. However, two very potent, selective PDE2 inhibitors developed by Bayer, Bay 60-7550 and Bay 31-9472, both with IC_{50} values of approx. 4 nM, have since become available and are beginning to be used for probing PDE2 function [70,71].

PDE2 is one of the principal PDEs that is expressed in platelets, where cGMP levels regulate its activity [72,73]. PDE2 has also been reported to represent, along with PDE4, one of the two major forms of PDE in murine thymocytes, where stimulation with PHA caused a transient reduction in PDE2 activity [74]. In contrast with murine thymocytes, PDE2 activity was not detected in resting human $CD19^+$ peripheral blood B-cells [54] or in purified human $CD4^+$ and $CD8^+$ T-lymphocytes [75]. PDE2A is a major PDE induced in monocytes cultured with M-CSF (macrophage colony-stimulating factor) to induce differentiation into macrophages [76].

PDE3

PDE3 is capable of hydrolysing both cAMP and cGMP; however, since the K_m for cGMP has generally been reported to be lower than that for cAMP, and the V_{max} is ten times greater for cAMP than for cGMP, cGMP readily inhibits the hydrolysis of cAMP by PDE3 by acting as a potent competitive inhibitor at the catalytic site [77–79]. Thus expression of PDE3 allows stimuli that elevate cGMP levels to augment cAMP-mediated signalling [78]. There are two PDE3 genes, PDE3A and PDE3B. Cilostazol is a PDE3 inhibitor that is a U.S. FDA (Food and Drug Administration)-approved therapy for claudication, owing to its activity on both platelets and endothelium [80]. Experimentally, milrinone, Org 9935, siguazodon, triquensin, motapizone and cilostamide have been used to specifically inhibit PDE3 isoforms, although it has been shown that milrinone, a commonly used PDE3 inhibitor is less selective for inhibition of PDE3 than cilostazol, and may have some PDE4 inhibitory activity (IC_{50} 16 μM) at the concentrations used (typically 1–10 μM) to test for PDE3 function [80,81]. Recently, a series of inhibitors showing considerable selectivity for PDE3B over that of PDE3A were developed [82].

In platelets, the predominant PDE3 isoform expressed is PDE3A. Although PDE2-like activity accounts for a greater proportion of basal platelet PDE activity, PDE3 plays a more critical role in regulating platelet aggregation [83]. Nitric oxide-induced cGMP synthesis induces platelet shape changes in a PKA-dependent manner, most likely by inhibiting the activity of PDE3A [84]. Mice rendered PDE3A-deficient by homologous recombination are viable but infertile as a result of an arrest of ovulated oocytes at the germinal vesicle stage. No comment was made of a bleeding diathesis in such PDE3A-knockout animals [85].

PDE3B is the predominant PDE3 isoform expressed in peripheral blood T-cells, where it appears to play a role in the heterologous desensitization processes that follow chronic elevation of cAMP levels [86,87]. Consistent with this, challenge of Jurkat, a cell line derived from T-ALL (acute lymphoblastic leukaemia) cells, with the adenylate cyclase activator forskolin induces both rapid and delayed augmentation of PDE3 activity [88]. Although generally not active alone, PDE3 inhibitors synergize with PDE4 inhibitors to inhibit Th1-mediated immune responses, block mixed leucocyte responses to major histocompatability antigens and suppress PHA- and anti-CD3-induced T-cell proliferation and IL-2 release [89–93]. Consequently, combined PDE3/4 inhibitors such as zardaverine have been developed [94,95]. Basal PDE3B is also present in human peripheral blood B-cells, where its expression can be up-regulated further in an apparent compensatory fashion following treatment with PDE4 inhibitors [96]. PDE3B is expressed in the K562 erythroid cell line, where it has been suggested to play a role in regulating foetal haemoglobin production [97].

In the myeloid cell line FDCP2, IL-4 induces PKB (protein kinase B)/Akt-dependent phosphorylation and activation of PDE3B [98,99]. Such a pathway is similar to that described for leptin-mediated signalling in the hypothalamus, where PI3K (phosphoinositide 3-kinase) activity results in activation of PDE3B and lowering of cAMP levels [100]. In one study, monocytes derived from atopic subjects showed a relative increase in levels of PDE3B [101], although, in another study, no difference was seen in the activity of PDE3 between normal and atopic subjects [102], and the functional effects of such elevation remain unclear. Within monocyte-derived macrophages, treatment with a combination of PDE3 and PDE4 inhibitors reduced TNF (tumour necrosis factor) secretion to a greater degree than either class of drugs alone [103]. A similar supra-additive effect was observed in relation to dendritic-cell-derived TNF production or in alveolar macrophage-induced arachidonate release [56,104]. In summary, drug therapies based on combined inhibition of PDE3 and PDE4 are likely to lead to more potent suppression of both T-cell and monocyte-mediated inflammatory signalling, an endpoint that may lead to both beneficial and potentially harmful clinical consequences.

PDE4

PDE4 enzymes are cAMP-specific and play an important role in the biology of haematopoietic cells. These PDEs all hydrolyse cAMP with K_m values in the range 1–4 μM. There are four human PDE4 genes (PDE4A–PDE4D), each of which has multiple splicing isoforms. PDE4 isoforms from all four subfamilies can be divided into 'long', 'short' and 'super-short' groups, depending on the presence or absence of two UCRs (upstream conserved regions) of sequence to the N-terminal side of the catalytic region, termed UCR1 and UCR2. The long forms contain both UCR1 and UCR2, the short forms contain only UCR2, and the super-short forms contain a truncated UCR2. There is also a form of PDE4, PDE4A7, which lacks both UCR1 and UCR2 and has a truncated catalytic region. PDE4A7 is widely expressed, but is catalytically inactive [105]. The function of this inactive form of PDE is unknown. The UCRs are intimately involved in regulating the activities of PDE4 isoforms. The UCR1 contains a serine residue at its extreme N-terminus whose phosphorylation by PKA augments catalytic activity of the PDE4 long forms [106]. PDEs 4B, 4C and 4D also possess a site for ERK phosphorylation near their C-terminal ends, and the presence or absence of UCRs, as well as the state of phosphorylation of UCR1 by PKA, determines the effects of ERK phosphorylation on PDE4 activity. In the absence of PKA phosphorylation, ERK inhibits PDE long forms. However, phosphorylation of PDE long forms in their UCR1

by PKA abolishes this inhibition by ERK. Since inhibition of PDE4 long forms by ERK would be expected to elevate cAMP levels and activate PKA to phosphorylate the PDEs, this no doubt accounts for the transient nature of ERK inhibition of PDE4 long forms. In contrast with PDE4 long forms, phosphorylation of PDE4 short forms by ERK results in their activation [106]. PDE4 splicing isoforms vary in their ability to dimerize, their subcellular distribution and their association with other signalling molecules such as β-arrestin and the immunophilin XAP2 (X-associated protein 2) [106–110]. One splice isoform, PDE4D3, has been shown to form a complex with the cAMP effector PKA as a result of association with an AKAP (A-kinase anchoring protein), with resultant cross-modulation of the activity of the kinase and the PDE [111]. The analysis of mice in which PDE4 genes have been deleted has confirmed that different PDE4 isoforms play markedly different roles in mammalian physiology. PDE4D-knockout mice have both impaired fertility and markedly reduced muscarinic agonist-induced tracheal smooth muscle contraction [112–114]. In contrast, PDE4B-knockout mice have a more than 90% reduction in LPS (lipopolysaccharide)-induced TNF production from circulating leucocytes [115] and more than 50% reduction from peritoneal macrophages [116]. Both isoforms play non-redundant roles in the recruitment of neutrophils to the lung in an endotoxin-inhalation model of lung injury [117].

As a result of the anti-inflammatory properties of PDE4 inhibitors, the pharmaceutical industry has devoted considerable effort to the clinical development of PDE4 inhibitors, with asthma or COPD (chronic obstructive pulmonary disease) as primary indications. Rolipram, one of the first specific inhibitors of PDE4, was studied in the 1980s as a potential therapy for depression or Parkinson's disease, but, despite some evidence of efficacy, development was halted because of the consistent ability of this drug to induce emesis at therapeutically relevant doses [118,119]. Such emetogenic effects of PDE4 inhibitors were ultimately linked to specific PDE4 isoforms, with PDE4D playing a predominant role [120]. More recently, two PDE4 inhibitors, cilomilast (GlaxoSmithKline) and roflumilast (Altana), have undergone clinical trials in asthma and COPD and have demonstrated significant activity in these illnesses [121–123]. Recently, using a novel scaffold-based drug-design method, PDE4 inhibitors were developed by Plexxikon which showed several-fold specificity for individual PDE4 subtypes within the PDE4 gene family [124]. Through use of this methodology, a PDE4 inhibitor, PLX513, was obtained which showed an approx. 11-fold selectivity for PDE4B over PDE4D, and this inhibitor was considerably more effective than rolipram in potentiating forskolin inhibition of the proliferation of lymphoma cell lines expressing high levels of PDE4B [125].

PDE4 inhibitors have important effects on the function of both B- and T-lymphocytes. In murine thymocytes, TCR (T-cell receptor) ligation led to an initial reduction followed by a sustained elevation in PDE4 activity [74]. Co-stimulation of human peripheral blood T-cells with anti-CD3 and anti-CD28 results in recruitment of PDE4 isoforms associated with the TCR–CD28 complex to lipid rafts, reducing local cAMP levels and augmenting TCR-mediated signalling [126]. Use of specific antisera identified concomitant recruitment to lipid rafts in such co-stimulated T-cells of a long PDE4A4 isoform, and short PDE4B2 and PDE4D1/D2 isoforms along with β-arrestin and G_i. Compartmentalization of PDEs to specific microdomains within the cell, along with other components of the cAMP signalling pathway, including adenylate cyclases, G-proteins and PKAs, is essential for controlling localized concentrations of cAMP [127–130]. As an example of the importance of such PDE localization in T-cell signalling, when full-length PDE4B2 is transfected into Jurkat cells, it associates with lipid rafts and enhances IL-2 production following TCR-stimulation, whereas transfection of a catalytically active, N-terminal truncated form of PDE4B2, unable to associate with lipid rafts, has no effect [131]. It is of note that, while PDE4B2 is the major form of PDE expressed in normal human T-cells, where it associates with the CD3ε chain of the TCR [132] and plays a critical role in regulating cytokine production during T-cell signalling [131], PDE4B is not expressed at all in the Jurkat [87,88], Molt4 [133] or CEM [134] T-leukaemic cell lines.

PDE4 inhibitors also reduce platelet activating factor, IL-8, IL-15 and SDF-1 (stromal cell-derived factor 1)-induced T-cell chemotaxis [135,136]. In a study of EAE (experimental autoimmune encephalomyelitis), rolipram blocked the production of Th1-type pro-inflammatory cytokines and the development of clinical signs of EAE in mice immunized with myelin basic protein [137]. PDEs 4A, 4B and 4D have been detected in primary human B-lymphocytes [54]. Smith et al. [125] detected the PDE4B2 transcript as the only PDE4B splice isoform expressed in primary naïve, centroblast, centrocyte and memory B-cells. Interestingly, PDE4B2 transcript levels were markedly lower in centroblast and centrocytes than in naïve B-cells or memory B-cells [125]. Stimulation of human B-cells with LPS and IL-4 augmented cAMP levels and reduced PDE4 activity by 50% [54].

Both monocytes and macrophages express PDE4A7, PDE4A10, PDE4B1, PDE4B2, PDE4C, PDE4D1 and PDE4D2 isoforms [138,139]. In contrast with monocytes, the predominant PDE4 isoform in immature and mature human dendritic cells is PDE4A [140]. Stimulation of T-cells with dendritic cells cultured in PDE4 inhibitors reduced their ability to induce a Th1 response, as judged by a reduction in interferon γ-producing T-cells [140]. In addition to their effects on T-cell and macrophage-mediated recruitment of neutrophils, PDE4 inhibitors have important effects on neutrophil function. The principal species expressed in human neutrophils is the 'short-form' PDE4B2 [141]. PDE4 inhibitors have been reported to inhibit neutrophil degranulation, superoxide generation, zymosan particle phagocytosis and zymosan-induced IL-8 secretion, particularly when combined with adrenergic stimuli such as the β-agonist salbutamol [142–144]. Cilomilast also reduces histamine release from human basophils [145]. In summary, PDE4 inhibitors suppress the function and inflammatory cytokine production of a wide variety of myeloid and lymphoid cells.

PDE5

PDE5, a cGMP-specific PDE family of considerable importance in regulating smooth muscle and endothelial cell function, is also present in platelets. Although having no effect on platelet function when used alone, PDE5 inhibitors augment nitroprusside's anti-platelet aggregation activity *in vitro* [146]. Although pre-treatment with the PDE5 inhibitor sildenafil has been reported to have significant anti-inflammatory effects in an animal model of airway hyper-reactivity [147], PDE5 activity has not been detected in human circulating T-cells, B-cells or monocytes [54].

PDE6

In adult human tissues, PDE6, a cGMP-specific PDE that is critical for rhodopsin signal transduction, is highly concentrated on internal membranes of retinal photoreceptors [148]. A single report has suggested that, in mouse F9 embryonal cells, PDE6 may also play a role as an effector for the Wnt (derived from segment polarity gene *wingless* in *Drosophila* and the proto-oncogene *int-1*)/Ca^{2+}/cGMP signalling pathway [149]. There have been no

publications documenting expression of PDE6 in the haematopoietic lineage.

PDE7

PDE7A and PDE7B are high-affinity cAMP-specific PDEs with K_m values for cAMP hydrolysis of approx. 0.2 μM (PDE7A) and 0.03–0.1 μM (PDE7B) [150–152]. PDE7 was first identified as a high-affinity cAMP PDE in human leukaemic T-cell lines that was insensitive to the PDE4 inhibitor, rolipram [153]. PDE7A1 expression has been detected in human T- and B-lymphocytes, monocytes, neutrophils and alveolar macrophages [91,154,155]. PDE4 and PDE7 both associate with a specific AKAP, myeloid translocation gene, in the Jurkat T-ALL cell line [156], again indicating the widespread nature of PDE compartmentalization. Co-stimulation of T-cells by cross-linking of CD3 and CD28 up-regulates the PDE7A1 and PDE7A3 isoforms [157,158]. Li et al. [157] reported that such PDE7A up-regulation was required for T-cell proliferation, as reduction of PDE7 expression with a PDE7A-specific antisense oligodeoxynucleotide inhibited T-cell proliferation in a manner that could be reversed with a PKA antagonist. However, anti-CD3/CD28-stimulated Th1 and Th2 cytokine production were reported recently to be normal in PDE7A-knockout mice, calling into question PDE7A's role in T-cell activation [159]. Surprisingly, antibody responses to keyhole-limpet haemocyanin are augmented in such mice.

The development of PDE7-specific inhibitors such as BRL 50481 has begun to clarify the potential clinical role of PDE7 inhibition in the treatment of inflammatory illnesses. In studies of $CD8^+$ T-cells, monocytes and lung macrophages, Smith et al. [160] found that BRL 50481 had little activity when used alone on freshly isolated cells. However, after 'aging' of monocytes in culture led to up-regulation of PDE7A1, BRL 50481 was able to block TNF secretion in a dose-dependent manner [160]. The PDE7 inhibitor also acted in at least an additive manner to reduce TNF secretion when combined with the PDE4 inhibitor rolipram [160]. Similarly, T-2585, a dual PDE4/PDE7 inhibitor, suppressed cytokine production and proliferation of activated human T-cells to a greater extent than that produced by RP 73401 (piclamilast), a PDE4-only selective inhibitor [161].

PDE8

PDE8A is a widely expressed high-affinity cAMP-specific PDE with highest expression levels in testis, ovary, small intestine and colon [162,163]. In contrast, reports on PDE8B expression are so far limited to the thyroid and brain [164,165]. PDE8 enzymes have quite high affinities for cAMP, with reported K_m values in the range 40–150 nM [162,163,166]. PDE8 is unique among PDE families in containing a PAS (Per-Arnt-Sim) domain in its N-terminal end. The function of this PAS domain in PDE8 is unknown, but inasmuch as the superfamily of proteins containing PAS domains function to sense changes in the cellular environment, frequently coupling signal transduction pathways to alterations in gene transcription [167,168], PDE8 may serve in some way as an extracellular environment sensor. In that regard, it is noteworthy that a recent report described the PAS-domain-dependent association of human PDE8A1 with IκB [inhibitor of NF-κB (nuclear factor κB)] proteins, and competition of the enzyme with NF-κB proteins for IκB binding. Binding of IκB to PDE8A stimulated its enzymatic activity 6-fold [169]. No PDE8-specific inhibitors have been reported, although dipyridamole, a non-selective inhibitor most potent on cGMP PDEs, does inhibit PDE8 [162,163,166]. PDE8 is notable in being insensitive to inhibition by methylxanthines such as IBMX (3-isobutyl-1-methylxanthine). PDE8A1, along with PDE7A3 and PDE7A1, has been reported to be induced in human T-lymphocytes stimulated with anti-CD3 and anti-CD28 antibodies [158], suggesting that PDE8A may play a role in activated lymphocytes, or in the activation process itself. PDE8A1 is constitutively expressed in the human T-cell line, Hut78 [158].

PDE9

PDE9A is a very-high-affinity cGMP-specific PDE that exhibits a K_m for cGMP of approx. 70 nM. PDE9A transcripts have been detected at highest levels in spleen, small intestine and brain [170–172]. While PDE9-specific inhibitors are not currently available, PDE9 is inhibited by drugs such as zaprinast and SCH51866, which are also known to be active on PDEs 1 and 5 [170,171]. PDE9, like PDE8, is insensitive to inhibition by methylxanthines. Despite the high level of expression in spleen, no reports examining PDE9 expression or function in haematopoietic cells have yet been published.

PDE10

PDE10 hydrolyses both cAMP and cGMP and contains GAF domains and a catalytic domain homologous with PDEs 2, 5 and 6 [173,174]. The K_m of PDE10 for cAMP (50 nM) is much lower than that for cGMP (3 μM), and the V_{max} for cGMP hydrolysis is 5-fold higher than for cAMP, leading to the suggestion that PDE10 may function as a cAMP-inhibited cGMP PDE [27]. The PDE10 transcript is expressed in heart, brain, placenta, testis and kidney, and PDE10 protein in pancreatic islets, but neither has been reported in cells of the haematopoietic lineage [173–175]. The observations that PDE10 is highly expressed in medium spiny neurons of the caudate and putamen regions of the striatum [176], that expression of PDE10 mRNA and protein decrease in transgenic mice expressing exon 1 of the human huntingtin gene [177,178], and that PDE10-knockout mice exhibit decreased locomotion and a synergistic effect on haloperidol-induced cataplexy (Frank Menniti, personal communication), suggest that PDE10 may function in control of motor movement. Inhibitor profiles of PDE10 have been reported [173,174,179]. The enzyme is sensitive to IBMX, and is inhibited by a number of agents that are known to inhibit other PDE families, such as vinpocetine, EHNA, rolipram, dipyridamole, sildenafil and zaprinast, but at concentrations at least severalfold greater. It has been observed, however, that papaverine, a compound used for decades as a general non-selective PDE inhibitor, potently inhibits PDE10 with an IC_{50} of approx. 30 nM, which is more than 10-fold lower than the concentration at which it inhibits PDEs from any other gene family [180,181]. Given its selectivity for PDE10 at low concentrations, papaverine is now being used to probe physiological functions for PDE10 [182].

PDE11

PDE11 is a widely expressed dual-substrate PDE that hydrolyses both substrates with K_m values in the range 0.5–6 μM [183–185]. The highest transcript levels of PDE11 are found in skeletal muscle, prostate, kidney, liver, pituitary, salivary glands and testis [184]. Like PDEs 2, 5, 6 and 10, PDE11 contains allosteric cGMP-binding GAF domains. However, splice variants of PDE11 exist which contain only a single GAF domain, whereas all forms of PDEs 2, 5, 6 and 10 identified so far contain two GAF domains [62]. PDE11-knockout mice have been generated, resulting in suppressed spermatogenesis and premature sperm capacitance,

suggesting a role for PDE11 in these processes; however, no gross abnormalities of these mice were seen, and no defects in haematopoietic cells were reported [186]. While PDE11 is sensitive to zaprinast, dipyridamole and IBMX, no specific PDE11 inhibitors have yet been identified. However, the clinically used inhibitor, tadalafil (Cialis), has dual PDE5/11 inhibitory properties and inhibits PDE11 with an IC_{50} of approx. 70 nM. The selectivity of tadalafil for inhibition of forms of PDE5 over that of forms of PDE11 has been reported variously as 5-, 14- and 40-fold [187,188]. No reports examining expression of PDE11 in haematopoietic cells have yet been published.

EFFECTS OF PDE INHIBITION IN SPECIFIC HAEMATOLOGICAL MALIGNANCIES

As is the case in most areas of cancer research, much of the work examining the role of PDE inhibitors as therapy for haematological malignancies has utilized *in vitro* studies of cell lines derived from patient samples. Such cell lines are likely to maintain some of the genetic alterations that are characteristic of the specific malignancy from which they were derived. Unfortunately, however, cell lines are also likely to differ significantly from such primary malignant cells as a result of the further genetic changes that occur during the selection process required in order to develop the cell line. As an example, primary malignant cells isolated from patients usually do not proliferate in tissue culture to any significant degree, while cell lines do. Nevertheless, for many haematological malignancies, it has been difficult or impossible to carry out extensive experimentation with primary malignant cells. For those illnesses where primary malignant cells are available in abundance, such as B-CLL (chronic lymphocytic leukaemia), it should be acknowledged further that circulating malignant cells may differ significantly from those that are in contact with non-malignant stromal cells in the bone marrow, lymph nodes and spleen, and that culture conditions *in vitro* differ dramatically from the complex signalling environment surrounding such malignant cells *in vivo*. These issues are likely to be of particular importance when studying PDE inhibitors, as the functional effects of these agents will in part reflect basal levels of adenylate or guanylate cyclase activity. With these caveats in mind, we will review what is currently known about the effects of PDE inhibitors on models of specific human haematological malignancies.

ALL

ALL is the most common form of leukaemia in children and young adults. Glucocorticoid therapy plays an important role in the successful treatment of ALL patients and *in vitro* glucocorticoid resistance of an ALL patient's lymphoblasts at presentation correlates with an increased risk of relapse following chemotherapy [189–197].

Jiang et al. [49] examined PDE activity in a human lymphoblastoid cell line, RPMI-8392, derived from a patient with ALL. PDE1B1 activity and transcript were detectable in the line, but not in resting HPBLs. Inhibition of PDE1 activity with the inhibitor vinpocetine (100 μM) induced apoptosis in RPMI-8392 cells, as well a variety of other cell lines in which the PDE1B transcript was detectable [49]. RPMI-8392 also underwent apoptosis after treatment with the PDE4 inhibitor either rolipram or RO 20-1724. To verify the specificity of the PDE1-inhibitor effect, the authors treated the cell line with an antisense oligodeoxynucleotide to PDE1B or a scrambled control oligodeoxynucleotide [49]. While the antisense oligodeoxynucleotide induced apoptosis and markedly reduced PDE1B transcript and enzymatic activity, the control oligodeoxynucleotide had none of these activities. Studies on primary ALL lymphoblasts were not performed. The clinical application of these observations remains unexplored.

While ALL cell lines are somewhat sensitive to the induction of apoptosis by agents that augment cAMP signalling, such compounds exert a more striking supra-additive apoptotic effect when combined with glucocorticoids. Myers et al. [198] have reported that treatment with the non-specific methylxanthine PDE inhibitor aminophylline (1,3-dimethylxanthine ethylenediamine) significantly prolonged the event-free survival of SCID (severe combined immunodeficient) mice injected with an ALL-derived pre-B-leukaemic cell line. In studies of the ALL cell line CEM by Ogawa et al. [199], addition of either aminophylline or the PDE4-specific inhibitor rolipram modestly augmented glucocorticoid-mediated apoptosis. Addition of the adenylate cyclase activator forskolin had a considerably more potent effect, particularly when combined with rolipram. Combined rolipram and forskolin treatment also led to up-regulation of p21 and p53 in CEM cells. Tiwari et al. [134], in contrast, found that treatment with rolipram caused no statistically significant augmentation of hydrocortisone or dexamethasone-induced apoptosis in CEM cells, while forskolin markedly augmented such glucocorticoid-induced apoptosis. In keeping with such an observation, forskolin, but not rolipram, augmented CEM cAMP levels. In summary, while it is clear that cAMP-mediated signalling can augment glucocorticoid-mediated apoptosis in the CEM ALL-like cell line, the role of PDE4 in ALL cell lines remains controversial, as does the relationship of CEM cell biology to that of primary ALL lymphoblasts. Future studies will need to be carried out using primary ALL lymphoblasts to determine whether PDE4 inhibitors may be clinically useful as a means by which to increase the efficacy of glucocorticoid therapy in this disease.

AML (acute myeloid leukaemia) and the role of cAMP in myeloid development

Traditionally, AML has been treated with cell-cycle-active drugs, such as cytosine arabinoside, and DNA-damaging agents, such as the anthracycline daunorubicin. However, a fundamental shift in the therapeutic approach to this illness occurred with the demonstration that those patients with APL (acute promyelocytic leukaemia), a subset of AML characterized by a 15:17 translocation involving the genes encoding the RAR (retinoic acid receptor) and a protein designated PML (promyelocytic leukaemia), can achieve a remission following treatment with an oral vitamin A derivative alone, ATRA (all-*trans*-retinoic acid) [200]. Instead of inducing apoptosis, by altering the transcriptional regulatory proteins associated with the mutant chimaeric RAR, ATRA releases a developmental arrest in the leukaemic blast cells, allowing the malignant promyelocytes to differentiate normally into mature neutrophils that have a lifespan measured in days. A second successful novel therapy for APL, arsenic trioxide, induces post-translational modifications of PML and apoptosis of APL blast cells, and is now approved for use in APL patients with relapses after ATRA therapy [201]. Recent work [206,208] suggests that agents that augment cAMP-mediated signalling, such as PDE inhibitors, may also relieve developmental arrest in APL and possibly other AML subtypes.

Early studies demonstrated that cAMP analogues, while inactive alone, increased retinoic-acid-induced differentiation of APL cell lines [202]. More recently, studies have focused on whether cAMP signalling can augment the ability of the approved therapies ATRA and arsenic trioxide to induce differentiation in APL blast cells. A cDNA microarray analysis

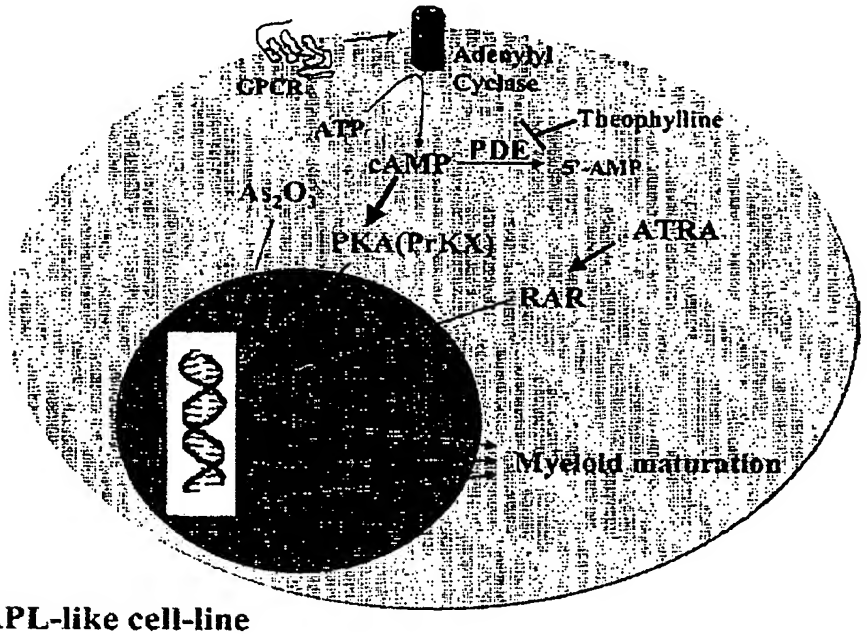

Figure 2 PDEs as targets for inducing maturation of myeloid cells

It is proposed that PDE inhibitors through their elevation of cAMP can drive the maturation of myeloid cells through the PKA-mediated regulation of transcription. In cell lines and in animal models, PDE inhibitors can synergize with either RAR agonists or arsenic trioxide to drive differentiation of AML blast cells. Of note, PrKX, a variant catalytic subunit of PKA that binds to RI (regulatory subunit of PKAI) but not to RII (regulatory subunit of PKAII), is expressed in myeloid cells and has been implicated in myeloid differentiation [304,305].

demonstrated that the PKA regulatory subunit α (PKA-RIα) gene was up-regulated following ATRA-induced differentiation of the APL cell line NB4 [203]. Treatment of either fresh APL blast cells or the NB4 cell line with ATRA rapidly increased cAMP levels and activated PKA. In another report, cAMP analogues were found to increase arsenic-trioxide-induced differentiation of both fresh APL cells and the cell lines NB4 and NB4-R1 (a derivative of NB4 resistant to retinoic acid-induced differentiation) [204]. In a gene expression-based high-throughput screening study, Golub and colleagues examined whether any of the 1739 currently FDA-approved or otherwise biologically well-characterized drugs would induce differentiation of acute myeloid blast cells [205]. Provocatively, five of the eight compounds identified as having differentiative activity [dimaprit dihydrochloride, pergolide methane sulphate, $R(-)$-apomorphine hydrochloride, EHNA hydrochloride and 16-oxoestradiol) had been previously reported to increase intracellular cAMP levels [205]. As at least one of these compounds, 16-oxoestradiol, induced maturation of AML blast cells from a patient with M1 AML, a subtype that is not ATRA-responsive, this study suggested that activation of cAMP signalling pathways may help to drive differentiation in AML subtypes for which differentiative therapies have not yet been identified [205].

Given the experiments cited above, it is not surprising that several groups have now focused on animal models to test the feasibility of improving differentiative therapy for AML with PDE inhibitors. Using PML/RARα transgenic mice as a model for treatment of APL, Guillemin et al. [206] reported that addition of a constant infusion of 8-Cl-cAMP to either retinoic acid or arsenic trioxide markedly improved clinical outcomes in such mice relative to use of the differentiative agents alone. As noted by the authors, however, the interpretation of such studies is complicated by the fact that 8-Cl-cAMP is metabolized *in vivo* to 8-chloro-adenosine, a highly active chemotherapeutic agent that in several reports has been argued to account for 8-Cl-cAMP's antineoplastic effects [207]. Guillemin et al. [206] reported further that addition of the non-specific PDE inhibitor theophylline (1,3-dimethylxanthine) to arsenic trioxide synergized in inducing blast cell differentiation in the murine APL model and restored normal haematopoiesis in an APL patient resistant to combined ATRA/arsenic trioxide therapy [206]. Parrella et al. [208] chose instead to investigate the potential utility of specifically inhibiting PDE4 in an *in vivo* model of APL. *In vitro*, the PDE4 inhibitor piclamilast induced PKA-mediated phosphorylation of RARα and PML/RARα in the NB4 APL cell line as well as ligand-dependent transactivation. *In vivo*, addition of piclamilast enhanced ATRA-induced maturation of NB4 leukaemia cells that had been implanted into the peritoneum of SCID mice. Piclamilast treatment also enhanced survival of such mice [208]. This study supports the concept of designing clinical trials to determine whether addition of PDE4 inhibitors may reverse resistance to ATRA and arsenic trioxide differentiative therapy in APL. Depiction of a model for targeting PDE as a means of inducing myeloid differentiation is presented in Figure 2.

In addition to driving differentiation in experimental models of myeloid leukaemia, and in contrast with the studies discussed below in which cAMP-mediated stimuli induce apoptosis in susceptible lymphoid cell populations, cAMP-mediated signalling confers an anti-apoptotic effect in many studies of myeloid lineage cells [209–212]. Both dibutyryl-cAMP and forskolin suppress spontaneous human neutrophil apoptosis; in some reports, by a PKA-independent mechanism [212,213]. In the promonocytic U-937 cell line, prior treatment with the non-specific PDE inhibitor theophylline in combination with forskolin

attenuates the induction of apoptosis by etoposide, camptothecin, heat shock, cadmium chloride or X-irradiation [209]. Although the precise mechanism by which cAMP-mediated signalling conferred an anti-apoptotic effect remains unclear, the authors did note that such signalling abrogated the expression of c-*myc*, and blocked etoposide-induced retinoblastoma dephosphorylation and PARP [poly(ADP-ribose) polymerase] cleavage. Similarly, in the promyelocytic HL60 cell line, forskolin, 8-Br-cAMP, the non-specific PDE inhibitor IBMX or the PDE3-specific inhibitor triquensin inhibited apoptosis induced by paclitaxel or thapsigargin [210]. Curiously, in this model system, the PDE4-specific inhibitors rolipram and RO 20-1724 induced apoptosis within 5 h, although this appeared to occur by a cAMP-independent mechanism. It was reported recently that these same PDE4 inhibitors, rolipram and RO 20-1724, when tested at high concentrations (100–500 μM), inhibited cyclo-oxygenase-2 expression in T-lymphocytes in a cAMP-independent manner by inhibiting the binding of NFAT (nuclear factor of activated T-cells) to DNA [214]. Inasmuch as Zhu et al. [210] used rolipram and RO 20-1724 at similarly high concentrations of 50–100 μM in their study, and inasmuch as other inhibitors of NFAT signalling, such as cyclosporin A [215] and FK506 [216], induce apoptosis of haematopoietic cells, it is quite possible that the anomalous effect of the PDE4 inhibitors on inducing apoptosis of HL60 cells seen by Zhu et al. [210] may have resulted from an inhibition of NFAT by these inhibitors, rather than an effect on PDE.

The opposing effects of cAMP-mediated signalling on the survival of specific lymphoid and myeloid cell subsets remains thus far largely unexplained. However, it should be noted that, while cAMP promotes apoptosis within lymphoid subsets, as well as some other cell types, including lung and mammary carcinoma cells [34], ovarian granulosa cells [217], fibroblasts [218] and primary cultured cardiomyocytes [219], a considerably wider range of cell lineages, including myeloid cells, pancreatic β-cells, hepatocytes, gastric and intestinal epithelial cells, and spinal motor, superior cervical ganglion sympathetic, dorsal root sensory, dopaminergic, cerebellar granule and septal cholinergic neurons, have been described in which cAMP signalling is anti-apoptotic [220–224]. Whether cAMP-mediated signalling will be pro- or anti-apoptotic, however, appears to depend on a number of different factors, including the nature of the stimulus, the nature of the cAMP effectors that predominate in the cell, the particular locale in which the cAMP signal occurs, and the strength of the signal. In mink lung epithelial cells for example, forskolin or 8-Br-cAMP markedly enhanced TGFβ1 (transforming growth factor β1)-induced apoptosis, but inhibited serum deprivation-induced apoptosis completely [225]. Additionally, whereas β-adrenergic agonists, 8-Br-cAMP and forskolin, inhibited spontaneous as well as Fas-antibody-induced apoptosis of eosinophils isolated from allergen-induced mouse lungs [226], treatment of eosinophils cultured with or without GM-CSF (granulocyte/macrophage colony-stimulating factor) with the PDE4 inhibitor, rolipram, induced apoptosis of these cells [227]. In pancreatic β-cells, forskolin or IBMX, which both produce high cAMP concentrations, as well as cAMP analogues themselves, have an anti-apoptotic effect via an EPAC (exchange protein activated by cAMP)-mediated pathway, whereas GLP-1 (glucagon-like peptide-1) and its receptor agonist, Exenatide, which produce a much more modest elevation of cAMP, produce their anti-apoptotic effect in these cells by activation of the PKA pathway [220].

Interestingly, in addition to being pharmacological targets for inducing or inhibiting apoptosis in some cell types, PDEs have also been shown to be caspase substrates. In rat-1 fibroblasts that undergo apoptosis in response to treatment with forskolin and IBMX, PDE4A5 is cleaved by caspase 3 during the apoptotic process [218]. Cleavage of PDE4A5 occurs in its unique N-terminal end, and this cleavage results in loss of the ability of PDE4A5 to complex with SH3 (Src homology 3)-domain-containing proteins, a dramatic change in the subcellular distribution of the enzyme, an increase in the catalytic activity of the cytosolic fraction of the enzyme, and a reduction in the catalytic activity of the particulate fraction of the enzyme. Further underscoring the different responses that can result from modulating cAMP in different subcellular compartments or microdomains within the cell, Huston et al. [218] observed that whereas overexpression of PDE4A5 in rat-1 fibroblasts protected them against staurosporine-induced apoptosis, overexpression of PDE4A8, which exhibits a very different subcellular distribution, potentiated staurosporine-induced apoptosis. In addition to PDE4A5, PDE5, PDE6 and, to some extent, PDE10, have been shown to be cleaved by caspase 3 during apoptosis as well [228,229]. In the case of PDE5, the cleavage occurs in the catalytic region and renders the enzyme completely inactive.

B-CLL: PDE4 as a therapeutic target

B-CLL, the most common leukaemia in adults, is a malignancy characterized by the gradual accumulation of clonal $CD5^+$, $CD19^+$ and $CD23^+$ mature-appearing B-cells in the bone marrow, spleen and lymph nodes, with resultant anaemia, thrombocytopenia, leucopenia, hypogammaglobulinaemia, infections and autoimmune disease. In early studies, culture with cAMP analogues induced B-CLL cell death [230,231]. However, a clinically feasible means of taking advantage of this observation was first proposed by Mentz et al. [232–234] in studies examining the effects of the non-specific PDE inhibitor theophylline on B-CLL cells *in vitro*. At doses of 50–100 μg/ml, theophylline, a commonly used therapy for asthma, augmented apoptosis in B-CLL cells [232]. At 4-fold lower doses, theophylline synergized with chlorambucil, an alkylator commonly used in the treatment of B-CLL [233]. Wiernik et al. [235] carried out a phase 2 trial of theophylline monotherapy in patients with early-stage B-CLL. Among 25 patients treated with 200 mg of theophylline given twice a day, there was one complete response and 18 other patients with stable disease. In simultaneous *in vitro* studies, the sensitivity of a patient's leukaemic cells to theophylline correlated with progression-free survival. Although this trial is certainly provocative, interpretation of a non-randomized study in early-stage B-CLL is hampered by the fact that, historically, only a subset of such B-CLL patients would be expected to progress clinically, even in the absence of therapeutic intervention. Theophylline has a narrow therapeutic window as high serum levels can induce tremulousness, nausea, vomiting and seizures. At therapeutic serum levels (5–20 μg/ml, corresponding to 25–100 μM), theophylline has only modest inhibitory effects on PDE isoforms *in vitro*. Mentz et al. [234] found that theophylline-induced apoptosis was only partially reversed by the cAMP enantiomeric antagonist Rp-8-Br-cAMPS, (8-bromoadenosine-3',5'-cyclic monophosphorothioate, Rp isomer). They also suggested that theophylline did not synergize with cAMP analogues in inducing apoptosis in B-CLL cells, although the concentration of theophylline used in these studies (100 μg/ml) was quite effective in inducing apoptosis by itself and therefore may have reduced the likelihood of observing synergistic interactions with forskolin.

As an alternative strategy, work in our laboratory has examined whether PDE family-specific inhibitors induce cAMP-mediated apoptosis of CLL cells. By Western blot analysis, B-CLL cells express a constitutive M_r 130000 form of PDE3 that co-migrates with recombinant PDE3B, a constitutive M_r 130000 form of PDE4A that co-migrates with recombinant PDE4A5, an

M_r 64000 rolipram-inducible form of PDE4B that co-migrates with recombinant PDE4B2, an M_r 70000 rolipram-inducible form of PDE4D that migrates slightly faster than recombinant PDE4D1, and a constitutive M_r 55000 form of PDE7 that co-migrates with recombinant PDE7A1 [96,154]. Although CLL samples have detectable PDE1B transcript, only a small subset of leukaemic samples express a constitutive M_r 74000 form of PDE1 by Western blot analysis that co-migrates with recombinant PDE1B, and enzymatic assays have not identified calmodulin-dependent or vinpocetine-sensitive PDE activity in B-CLL samples to date [236].

To assess the potential ability of family-specific PDE inhibitors to induce apoptosis in B-CLL, leukaemic samples were exposed for up to 3 days to inhibitors, followed by a Hoechst 33342 FACS-based apoptosis assay [236]. The PDE1 inhibitor, vinpocetine, induced apoptosis at 10 μM and 30 μM, but not at 2 μM. Given the absence of detectable PDE1 enzymatic activity in B-CLL cells, the possibility of 'off-target' effects for vinpocetine is of concern. The PDE3-specific inhibitor cilostamide did not induce apoptosis. In contrast, in ten of 14 CLL samples tested, treatment with 10 μM rolipram, a PDE4-specific inhibitor, induced apoptosis in at least a third of the leukaemic CLL cells with a range of 38–80% of absolute apoptotic cells. Treatment with forskolin (40 μM), an adenylate cyclase activator, had significantly less apoptotic activity than treatment with rolipram, suggesting that adenylate cyclase activity is already of a sufficient magnitude in B-CLL cells *in vitro* that basal PDE4 activity is required to regulate cAMP-mediated signalling, which unchecked leads to apoptosis. The induction of apoptosis by PDE4 inhibitors was significantly slower than that observed for many other apoptotic stimuli, with the majority of apoptosis occurring between 24 and 48 h after the addition of the drug. Treatment with rolipram was associated with elevation of cAMP levels as well as dramatic up-regulation of PDE4B2 transcript and protein levels as a compensatory feedback mechanism [96,236]. Consistent with known defects in CLL B-cell receptor signalling, cross-linking of cell-surface immunoglobulin protected normal circulating B-cells from PDE4-induced apoptosis, but had no such protective effect in B-CLL cells.

A growing literature suggests that combined inhibition of multiple PDE families may be more efficacious than targeted therapy of a single PDE family. In keeping with these observations, we found that inhibition of PDE4 in B-CLL cells *in vitro* led to augmented levels of PDE3B by Western blot analysis, presumably as part of a compensatory feedback loop in response to increased cAMP signalling induced by the PDE4 inhibitor [96]. Although, as noted above, the PDE3 inhibitor cilostamide had no apoptotic effects when used alone, when combined with rolipram, addition of cilostamide (1 μM) augmented apoptosis in leukaemic cells from five of seven patients 'resistant' (defined as less than 50% apoptosis after treatment with 10 μM rolipram) to the induction of apoptosis by rolipram alone. Thus combination therapy with PDE3 inhibitors and PDE4 inhibitors or use of dual-selective drugs may be of use in a subset of relatively PDE4 inhibitor resistant B-CLL patients.

Sarfati et al. [237] reported that CLL cells were sensitive to the induction of apoptosis by the PDE5 and PDE6 inhibitors sildenafil, vardenafil and methoxyquinazoline. As the authors were unable to detect PDE5, but did detect PDE6, expression in B-CLL cells by microarray analysis, they suggest that aberrant expression of PDE6 may account for the sensitivity of B-CLL cells to these PDE inhibitors [237]. However, the apoptosis EC_{50} values reported by the authors match the IC_{50} values of these drugs for PDE4 to a much closer degree than for PDE5 or PDE6 [237]. For example, vardenafil (EC_{50} 1.5 μM) has been reported to have an IC_{50} of 0.81 nM for PDE5 and 1.91 μM for PDE4B [238]. Similarly, sildenafil (EC_{50} 4.1 μM) has an IC_{50} of 7.68 μM for PDE4, but 3.5 or 33 nM for PDE5 and PDE6 respectively [239]. Interestingly, the PDE5 inhibitor zaprinast (IC_{50} of approx. 0.2–0.3 μM for both PDE5 and PDE6), which the authors reported was completely ineffective in inducing apoptosis in CLL cells, is also completely inactive against recombinant PDE4A [240–243]. Thus it would appear that the true therapeutic target of these PDE5 inhibitors at these concentrations in B-CLL is PDE4.

B-CLL cells also express PDE7A by Western blot analysis [96]. Although regulation of PDE7 by cAMP has been documented in a human B-cell line, the absence until recently of PDE7-specific inhibitors that lack significant off-target effects has hampered evaluation of PDE7 as a therapeutic target in this disease [154].

DLBCL (diffuse large B-cell lymphoma): PDE4B as a prognostic factor

DLBCL accounts for approx. one-third of non-Hodgkin's lymphomas. Among patients with advanced DLBCL treated with standard multiagent chemotherapy regimens that contain an alkylator, an anthracycline, a vinca alkaloid and a glucocorticoid, roughly 60% will relapse within 3 years. In 2002, Shipp et al. [244] published a study in which they prospectively assessed diagnostic tumour specimens from 77 DLBCL patients with oligonucleotide microarrays in an effort to identify genes whose expression was predictive of relapse. A predictor containing 13 genes was ultimately identified that correctly classified 32 patients with cured disease from 26 patients with fatal or refractory disease. One of the 13 genes whose expression conferred a poor prognosis was PDE4B. Shipp et al. [244] then performed a retrospective analysis of an independent microarray study of newly diagnosed DLBCL patients by Alizadeh et al. [245] and identified a similar correlation between PDE4B expression and relapse, albeit this was not statistically significant in this smaller study ($P = 0.07$). In a subsequent confirmatory study, this group once again observed a statistically significant correlation between PDE4B expression and relapse in set of 112 previously untreated DLBCL patients (57 cured patients and 55 patients with fatal/refractory disease) [125].

The demonstration that PDE4B transcript expression in DLBCL tumour specimens correlates with higher risk of relapse following standard chemotherapy is of great interest, but could potentially reflect quite divergent underlying signalling states in DLBCL tumour specimens. PDE4B transcript levels are augmented as a negative-feedback loop following elevation of intracellular cAMP levels, so DLBCL tumour cells that will ultimately prove refractory to traditional chemotherapy may have such augmented cAMP levels. Such an elevation in intracellular cAMP levels could either be due to an intrinsic alteration in the production or catabolism of cAMP or reflect augmented extracellular signals that activate adenylate cyclase. Alternatively, using a 'selection' rather than a 'compensation' model, PDE4B transcript levels may be higher in resistant DLBCL tumour samples because chemotherapy selected a subset of DLBCL cells that expressed higher levels of PDE4B and were protected from chemotherapy-induced apoptosis as a result of lower cAMP and PKA-mediated signalling (see below). In keeping with this latter hypothesis, it is of interest that Shipp et al. [244] identified high expression of NOR1 (NR4A1), a member of the nerve growth factor 1B family, as correlating significantly with cure in DLBCL, both in their database and that of Alizadeh et al. [245]. As expression of NOR1 is linked to PKA-mediated CREB [CRE (cAMP-response element)-binding protein] activation, DLBCL sensitivity to chemotherapy may correlate with concurrent

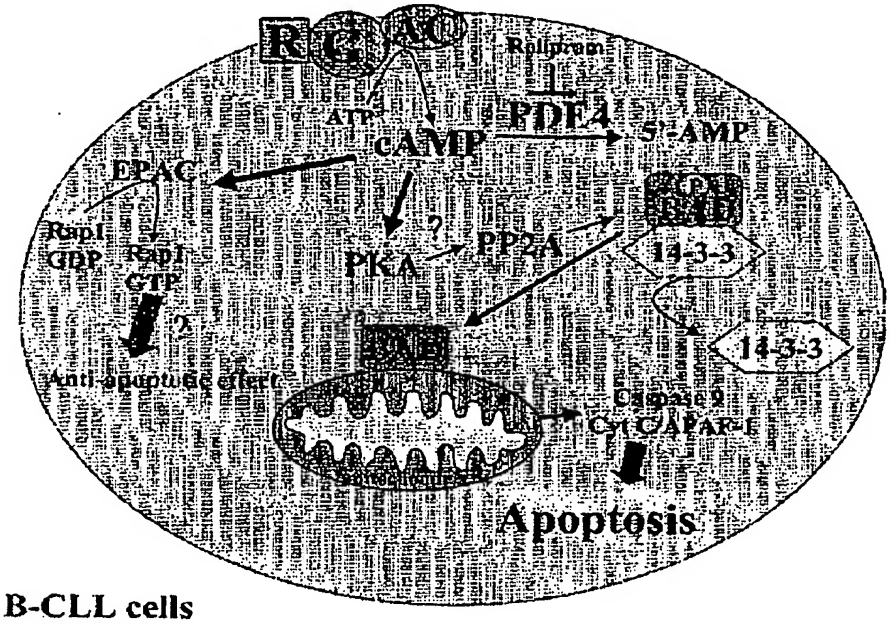

Figure 3 PDE4 as a target for inducing apoptosis in B-CLL cells

PDE4-inhibitor-induced apoptosis in B-CLL cells appears to occur by a PKA-mediated pathway, as the percentage of cells that undergo apoptosis following rolipram treatment is reduced by co-treatment with the PKA antagonist Rp-8-Br-cAMPS. In contrast, activation of the cAMP effector EPAC1 by the EPAC-specific agonist 8-CPT-2Me-cAMP is anti-apoptotic in B-CLL cells. As the net effect of PDE4 inhibitor treatment is to induce apoptosis in B-CLL cells, the pro-apoptotic PKA-mediated pathway appears to predominate over the anti-apoptotic effects of PDE4-inhibitor-mediated EPAC activation. One study reported that PKA signalling induces apoptosis in B-CLL cells by activating PP2A, which in turn dephosphorylates BAD, releasing it from 14-3-3 proteins, and allowing BAD to initiate apoptosis through a mitochondrial pathway. Cyt. C, cytochrome *c*.

PKA-mediated CREB activation, a process that could, conversely, be inhibited in tumour cells that express elevated PDE4B levels [246]. Finally, it is formally possible that elevated PDE4B transcripts identified in DLBCL patients with poor prognosis could derive from non-malignant cells in the tumour specimen. Of note, a recent gene expression profiling experiment carried out in follicular lymphoma demonstrated that prognosis for such patients correlated with the molecular features of the non-malignant immune cells, probably T-cells and macrophages, that are present in the lymphoma tumour specimens at diagnosis [247].

MECHANISMS OF PDE-INHIBITOR-INDUCED APOPTOSIS

PDE4-inhibitor-induced BAD dephosphorylation in B-CLL

Dose–titration studies in our laboratory with the PDE4 inhibitor rolipram in primary CLL cells have documented that, unlike prior work in the same cell type with theophylline, a good correlation exists between the degree to which a given concentration of this drug augments levels of intracellular cAMP and the percentage of leukaemic cells induced to undergo apoptosis ($r^2 = 0.998$) [35,234]. Rolipram treatment induces mitochondrial depolarization, cytochrome *c* release and caspase 9 and 3 cleavage in B-CLL cells [248,249]. Consistent with the hypothesis that such a mitochondrial pathway of apoptosis is critical in rolipram's effects on CLL cells, inhibitors of caspase 9, but not caspase 8, block rolipram-induced apoptosis [249]. Studies have therefore focused on the mechanism by which PDE4-inhibitor-induced augmentation of intracellular cAMP levels results in mitochondrial release of cytochrome *c*. Co-treatment of B-CLL cells with the enantiomeric cAMP analogue, Rp-8-Br-cAMPS, a known PKA inhibitor, reduces rolipram-mediated apoptosis, suggesting that rolipram induces apoptosis in this cell type through PKA [250].

Rolipram treatment reduces Bcl-2 and Bcl-X_L levels and augments Bax levels, suggesting that an alteration in the relative levels of pro- and anti-apoptotic Bcl-2 family members may play a role in triggering cytochrome *c* release [248]. Rolipram induces Ser^{112} dephosphorylation of the pro-apoptotic BH3-only Bcl-2 family member BAD [Bcl-2 (B-cell lymphoma 2 anti-apoptotic protein)/Bcl-X_L-antagonist, causing cell death] in CLL cells, with resulting loss of cytosolic sequestration with 14-3-3 protein and translocation of BAD to mitochondria [249]. Rolipram treatment of B-CLL cells induces up-regulation of PP2A (protein phosphatase 2A)-like enzymatic activity and PP2A catalytic subunit by Western blot analysis. Okadaic acid, a PP2A inhibitor, reduced BAD Ser^{112} dephosphorylation and rolipram-induced apoptosis. These studies suggest that PKA may, by an as yet undetermined mechanism, augment PP2A expression and activity in B-CLL cells, resulting in BAD dephosphorylation and the induction of a mitochondrial pathway of apoptosis. A model by which PDE4 may serve as a target for inducing apoptosis in B-CLL cells based on these results is presented in Figure 3.

Role of EPAC in PDE4-inhibitor-induced apoptosis in B-CLL

In 1998, two groups identified a novel family of Rap1 EPACs (EPAC1 and EPAC2) or cAMP-GEFs (cAMP-activated guanine nucleotide-exchange factors) [251,252]. Subsequent studies identified a cAMP analogue, 8-CPT-2Me-cAMP [8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate],

that potently activates the EPAC enzymes, but not PKA [253]. Studies using this compound have implicated EPAC in integrin-mediated adhesion in epithelial cells and in pancreatic β-cell exocytosis [254,255]. In oligonucleotide array studies, the EPAC1 transcript was expressed at high levels in CLL cells [256]. Tiwari et al. [250] reported that, while the EPAC1 (but not EPAC2) transcript was detectable in normal circulating B-cells and CLL cells, it was undetectable in circulating human neutrophils, monocytes or T-cells. Using real-time PCR, EPAC1 transcript levels were 50-fold higher in B-CLL cells than in normal or activated B-cells. Treatment with the PDE4 inhibitor rolipram in combination with forskolin or even as a single agent led to robust Rap1 activation in CLL cells, but not in normal or activated B-cells, T-cells, neutrophils or monocytes. The hypothesis that such PDE4-inhibitor-induced Rap1 activation was due to EPAC was confirmed by the demonstration that 8-CPT-2Me-cAMP also robustly activated Rap1 in CLL cells, but not in any of the other populations described above.

While rolipram treatment led to both PKA activation (as judged by CREB phosphorylation) and Rap1 activation in B-CLL cells, 8-CPT-2Me-cAMP treatment, as expected, led only to Rap1 activation, allowing an analysis of whether PDE4 inhibitors induce apoptosis through a PKA or EPAC-mediated pathway. Surprisingly, activation of EPAC with 8-CPT-2Me-cAMP reduced basal apoptosis in B-CLL cells. Thus, in B-CLL cells, it appears that PDE4 inhibitor treatment activates two pathways, a pro-apoptotic PKA-mediated pathway whose effects predominate over anti-apoptotic effects mediated by EPAC1 (see Figure 3). Although the mechanism by which EPAC1 activation confers such an effect in B-CLL remains undetermined, it is notable that two other groups examining pancreatic β-cells and hepatocytes have also reported 8-CPT-2Me-cAMP to be anti-apoptotic [220,221]. In the hepatocyte study, Webster and colleagues reported that EPAC activation is anti-apoptotic as a result of PI3K and PKB/Akt activation [221].

Role of GR (glucocorticoid receptor) signalling in PDE4-inhibitor-induced apoptosis

Those subsets of lymphoid cells that are sensitive to cAMP-mediated apoptosis tend also to be sensitive to glucocorticoid-mediated apoptosis. While the human T-ALL cell line CEM undergoes apoptosis following exposure to cAMP analogues, McConkey and colleagues observed that a GR-defective variant of this cell line, ICR.27, was resistant to cAMP-mediated apoptosis. Stable transfection of ICR.27 with GR restored sensitivity to the cAMP analogues, suggesting that cAMP-induced apoptosis requires at least some aspect of GR function [257]. Consistent with such 'cross-talk', the catalytic subunit of PKA has been reported to associate with the GR [258].

cAMP signalling also augments glucocorticoid-induced apoptosis, a potentially important phenomenon as glucocorticoids continue to play a key role in chemotherapeutic regimens for ALL, DLBCL and multiple myeloma [199,259–261]. In a study of primary leukaemic cells from patients with B-CLL, Tiwari et al. [134] identified PDE4 inhibitors as a clinically feasible means by which to take advantage of such enhancement of glucocorticoid-mediated apoptosis by cAMP signalling. Rolipram or RO 20-1724 synergized with either hydrocortisone or dexamethasone in inducing apoptosis in B-CLL cells but not primary T-cells. As had previously been reported in studies of glioma cells, rolipram treatment of primary B-CLL cells enhanced hydrocortisone-induced transactivation of transiently transfected GRE (glucocorticoid-responsive element) promoter-containing luciferase constructs [262]. Prior treatment with the PKA antagonist Rp-8-Br-cAMPS markedly reduced glucocorticoid-mediated apoptosis in B-CLL cells, suggesting that the basal level of PKA activity in leukaemic cells may dictate the effectiveness with which glucocorticoid therapy induces apoptosis in such cells. A model depicting the synergistic effects of glucocorticoids and PDE4 inhibitors on induction of B-CLL cell apoptosis is presented in Figure 4.

Ideally, it would be of interest to similarly determine whether modulation of GR function with a GR antagonist alters the ability of PDE4 inhibitors to induce apoptosis in B-CLL. Unfortunately, in findings similar to those reported by Gruol and Altschmied [260] in immature murine T-cells, while the best characterized GR antagonist mefipristone (RU 486) had no activity when used alone on B-CLL cells, it acted as a GR agonist when combined with either rolipram or other drugs that activate PKA signalling. Refojo et al. [263] have argued that cAMP-mediated potentiation of glucocorticoid-mediated apoptosis is independent of CRE transcriptional activation as it was unaffected by co-transfection of CRE 'decoy' oligonucleotides that effectively inhibited transcription of *bona fide* CRE-containing promoters [263]. In aggregate, these studies support clinical trials to investigate whether the addition of PDE4 inhibitors to chemotherapeutic regimens in which glucocorticoids play an important role will enhance the efficacy of such therapies.

If cAMP and PDE4 inhibitor-mediated apoptosis is in fact mediated to an important degree through the activity of the GR, the mechanism by which glucocorticoids themselves induce apoptosis remains a subject of some controversy. Although the GR was initially characterized as a DNA-binding transcription factor that was capable of activating GRE-containing promoters, the medically beneficial anti-inflammatory activity of glucocorticoids appears to be the result of GR-mediated 'transrepression' of signalling by a variety of other transcription factors such as AP-1 (activator protein 1) and NF-κB though a tethering mechanism that is independent of the GR's DNA-binding activity [264]. Thymocytes from 'knockin' mice that express the A458T GR are unable to transactivate GRE-containing promoters as a result of a point mutation that abrogates dimerization of the GR. Thymocytes from such 'GR^{dim}' mice are resistant to glucocorticoid-mediated apoptosis, suggesting that, at least in this normal lymphoid population, glucocorticoid-mediated apoptosis is mediated by the GR's transactivation activity [265]. In contrast, glucocorticoid-mediated apoptosis of cell lines derived from lymphoid malignancies appears to be dependent upon the transrepressive activity of the GR [266]. Whether, with regard to the mechanism by which steroids (and PDE4 inhibitors) induce apoptosis, primary malignant lymphoid cells such as CLL cells resemble to a greater degree normal thymocytes or cell lines derived from malignant cells will require experimental techniques that allow one to address these questions within a primary malignant cell population.

The synergistic effect of glucocorticoid and cAMP signalling pathways on apoptosis may also result, in part, from effects of glucocorticoids on the expression and regulation of PDEs. Early studies on cultured hepatoma cells showed that long-term incubation of cells with dexamethasone led to a 25–40 % decrease in PDE activity and markedly enhanced the elevation of cAMP in the cells in response to adrenaline and theophylline [267]. Subsequent studies with cultured rat hepatocytes showed that dexamethasone selectively reduced the transcription of PDE4D [268]. Similarly, a recent study in human osteosarcoma cells showed that long-term treatment with dexamethasone selectively decreases the mRNA of PDE4A4 and PDE4B1 by 50–70 % [269]. In human T-lymphocytes, beclomethasone attenuated the long-term induction of PDE activity that occurs following activation by PHA or IL-2 [270]. Long-term incubation of murine T-cell

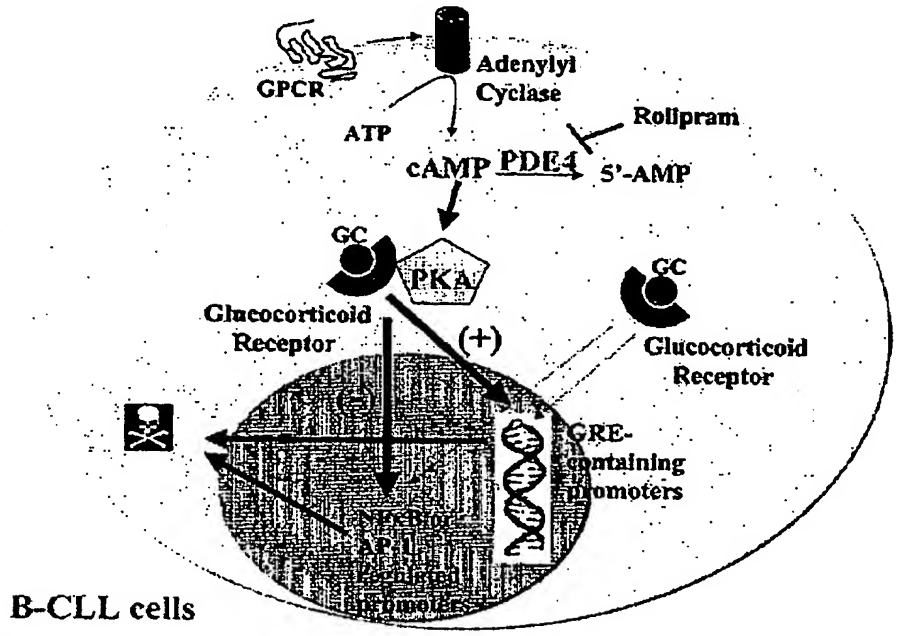

Figure 4 PDE4 inhibitors augment glucocorticoid-induced apoptosis of B-CLL cells

In B-CLL cells that are relatively resistant to apoptosis induced by either hydrocortisone or dexamethasone, co-treatment with rolipram can increase the percentage of apoptotic cells in a supra-additive manner. Experiments with GRE-containing luciferase reporter constructs demonstrate that rolipram treatment also augments glucocorticoid-mediated apoptosis and glucocorticoic-induced GRE activation in B-CLL cells, suggesting that levels of PKA activity may serve as a 'rheostat' that determines the apoptotic outcome of glucocorticoid therapy in this B-cell malignancy. Although the precise mechanism by which PKA activity modulates glucocorticoid-induced apoptosis remains unknown, the catalytic subunit of PKA has been reported to associate with the GR and to phosphorylate co-activators or co-receptors that are associated with this transcriptional complex. Notably, the mechanism by which GR signalling induces apoptosis in lymphoid cells remains controversial. GR-mediated apoptosis could involve either transactivation of GRE-containing promoters or GR-mediated *trans*-repression of other transcription factors such as NF-κB or AP-1 (activator protein 1) through a tethering mechanism that does not require a functional GR DNA-binding domain. GC, glucocorticoid; GPCR, G-protein-coupled receptor.

hybridomas with dexamethasone results in a 32–65% decrease in PDE activity and a 5-fold increase in cAMP levels [271]. Hence, multiple effects of glucocorticoids on cAMP signalling may account for the observed synergism between these two signalling pathways, including effects of glucocorticoids on PDE expression.

DLBCL: cAMP-mediated PI3K and Akt activation

To examine the explanation for the correlation between high PDE4B transcript levels in newly diagnosed DLBCL tumour specimens and relapse after chemotherapy, Smith et al. [125] carried out a study with DLBCL cell lines. Transfection of DHL6, a line with low basal PDE4B expression, with a PDE4B2 expression construct reduced intracellular cAMP levels after treatment with forskolin (40 μM) and protected these cells against forskolin-induced growth arrest and apoptosis. Surprisingly, PCR analysis and experiments performed with the PKA inhibitors H-89 and a PKI (cAMP-dependent protein kinase inhibitor)-derived peptide suggested that neither PKA nor EPAC are relevant effector proteins of forskolin-mediated apoptosis in this model system.

Smith et al. [125] detected a marked reduction in Akt Ser^{473} phosphorylation in rolipram/forskolin-treated DHL6 cells with maintenance of total Akt levels, while such a reduction in Akt phosphorylation was not detected in DHL6 PDE4B stable transfectants following the same treatment. As incubation of DHL6 cells with rolipram and forskolin also markedly reduced levels of PtdIns(3,4,5)P_3, the product of PI3K, the authors hypothesize that cAMP-mediated signalling by an as yet uncharacterized effector protein leads directly or indirectly to inhibition of PI3K activity, with a resultant reduction in Akt phosphorylation. The authors conclude that co-treatment with PDE4B and PI3K/Akt inhibitors may serve to reduce resistance to apoptosis in DLBCL. These results are depicted in a model presented in Figure 5.

Specificity of PDE4-inhibitor-induced apoptosis

cAMP-induced apoptosis is limited to small subsets of normal lymphoid cells, lymphoid cell lines and lymphoid neoplasms. PDE4-inhibitor-induced apoptosis occurs within a yet smaller subset of these cAMP-sensitive lymphoid cell populations. What accounts for the specificity of PDE4-inhibitor-induced apoptosis? One potential mechanism for such specificity might theoretically be that of so-called 'flux-mediated sensitivity' [24], meaning that those cell populations with robust basal adenylate cyclase activity would be markedly more sensitive to cyclic nucleotide PDE inhibitors than cells in which the basal rate of synthesis of cAMP is low. While, from limited data, it does appear that treatment of B-CLL cells with PDE4 inhibitors alone leads to a greater elevation in intracellular cAMP levels than comparable treatment of whole mononuclear cells, a predominantly T-cell population, it does not appear that this accounts for the relative sensitivity of B-CLL cells to PDE4 inhibitors [236]. Thus treatment of either isolated T-cells or whole mononuclear cells with a combination of rolipram and forskolin results in markedly higher levels of intracellular cAMP than those observed in CLL cells treated with PDE4 inhibitors alone, yet the rate of apoptosis

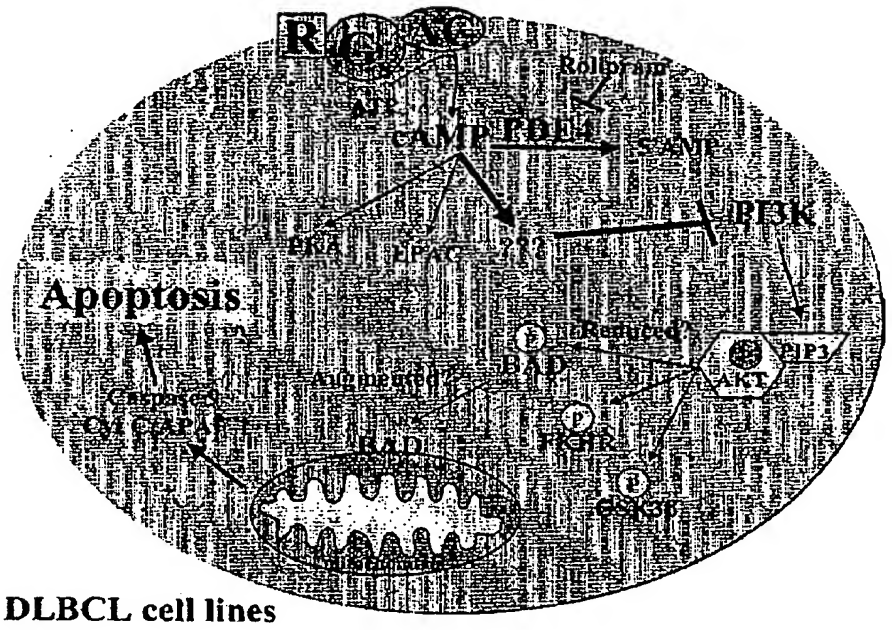

Figure 5 PDE4 inhibitors induce apoptosis in DLBCL cell lines

Elevated levels of PDE4B2 protect DLBCL cell lines from forskolin-induced apoptosis, and PDE4B-selective inhibitors overcome this, allowing forskolin to elevate cAMP levels and induce apoptosis. In this case, cAMP was reported to act neither through EPAC nor through PKA, but instead through an as yet undefined effector to inhibit PI3K, thereby reducing PKB/Akt activity and consequent phosphorylation of BAD. Dephosphorylated BAD can then induce apoptosis through the mitochondrial pathway. Alteration of the phosphorylation state of other PKB/Akt phosphorylation targets such as FKHR (forkhead in rhabdomyosarcoma) [306] and GSK3β (glycogen synthase kinase 3β) [307] may also contribute. Cyt C, cytochrome c; PIP3, PtdIns(3,4,5)P_3.

induced in such rolipram/forskolin-treated T-cells remains very low [236]. Thus levels of total intracellular cAMP clearly do not mirror rates of apoptosis in sensitive and resistant lymphoid populations.

Alternatively, the unique sensitivity of specific lymphoid subsets to PDE4 inhibitors could be accounted for by the expression of particular PDE4 isoforms that regulate cAMP signalling pathways whose unfettered activity triggers apoptosis. This hypothesis also does not hold up under scrutiny, as the PDE4 isoforms expressed in B-CLL cells; PDE4A5, PDE4B2 and PDE4D1/D2, have all been reported in PDE4 inhibitor-resistant cell types, such as T-cells. It should be noted, however, that this summary does not take into account the basal levels of PDE4 isoforms expressed in such cells before challenge with a cAMP signalling stimulus. The work by Smith et al. [125] in DLBCL cell lines has demonstrated that elevating basal levels of PDE4B2 protect cell lines that are initially sensitive to forskolin-induced apoptosis. Whether such forskolin-induced cAMP signalling in cell lines sheds light on the apoptosis induced by treatment with the PDE4 inhibitor alone in susceptible primary lymphoid cells remains to be seen.

Thus, while elevation of intracellular cAMP levels and the presence of particular PDE4 isoforms may both be necessary for PDE4-inhibitor-induced apoptosis, they are not sufficient. The specificity of this form of apoptosis lies downstream, either potentially in the specific subcellular organization of the effectors and regulators of cAMP-mediated signalling in these lymphoid cells or in the specific targets of PKA or other unidentified effector proteins. As noted above, the details of such downstream pro-apoptotic signalling pathways remain controversial and beckon further exploration.

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REFERENCES

1 Rall, T. W. and Sutherland, E. W. (1958) Formation of a cyclic adenine ribonucleotide by tissue particles. J. Biol. Chem. **232**, 1065–1076
2 Butcher, R. W. and Sutherland, E. W. (1962) Adenosine 3',5'-phosphate in biological materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-phosphate in human urine. J. Biol. Chem. **237**, 1244–1250
3 Robison, G. A., Butcher, R. W., Oye, I., Morgan, H. E. and Sutherland, E. W. (1965) The effect of epinephrine on adenosine 3',5'-phosphate levels in the isolated perfused rat heart. Mol. Pharmacol. **1**, 168–177
4 Abell, C. W. and Monahan, T. M. (1973) The role of adenosine 3',5'-cyclic monophosphate in the regulation of mammalian cell division. J. Cell Biol. **59**, 549–558
5 Pastan, I. H., Johnson, G. S. and Anderson, W. B. (1975) Role of cyclic nucleotides in growth control. Annu. Rev. Biochem. **44**, 491–522
6 Friedman, D. L. (1976) Role of cyclic nucleotides in cell growth and differentiation. Physiol. Rev. **56**, 652–708
7 Franks, D. J., MacManus, J. P. and Whitfield, J. F. (1971) The effect of prostaglandins on cyclic AMP production and cell proliferation in thymic lymphocytes. Biochem. Biophys. Res. Commun. **44**, 1177–1183
8 MacManus, J. P., Whitfield, J. F. and Youdale, T. (1971) Stimulation by epinephrine of adenyl cyclase activity, cyclic AMP formation, DNA synthesis and cell proliferation in populations of rat thymic lymphocytes. J. Cell. Physiol. **77**, 103–116
9 Ralph, P., Hyman, R., Epstein, R., Nakoinz, I. and Cohn, M. (1973) Independence of theta and TL surface antigens and killing by thymidine, cortisol, phytohemagglutinin, and cyclic AMP in a murine lymphoma. Biochem. Biophys. Res. Commun. **55**, 1085–1091
10 Coffino, P., Bourne, H. R. and Tomkins, G. M. (1975) Mechanism of lymphoma cell death induced by cyclic AMP. Am. J. Pathol. **81**, 199–204

11 Thompson, W. J. and Appleman, M. M. (1971) Multiple cyclic nucleotide phosphodiesterase activities from rat brain. Biochemistry **10**, 311–316
12 Weiss, B. and Hait, W. N. (1977) Selective cyclic nucleotide phosphodiesterase inhibitors as potential therapeutic agents. Annu. Rev. Pharmacol. Toxicol. **17**, 441–477
13 Kramer, G. L., Garst, J. E., Mitchel, S. S. and Wells, J. N. (1977) Selective inhibition of cyclic nucleotide phosphodiesterases by analogues of 1-methyl-3-isobutylxanthine. Biochemistry **16**, 3316–3321
14 Weiss, B. (1975) Differential activation and inhibition of the multiple forms of cyclic nucleotide phosphodiesterase. Adv. Cyclic Nucleotide Res. **5**, 195–211
15 Epstein, P. M., Fiss, K., Hachisu, R. and Andrenyak, D. M. (1982) Interaction of calcium antagonists with cyclic AMP phosphodiesterases and calmodulin. Biochem. Biophys. Res. Commun. **105**, 1142–1149
16 Hirschhorn, R., Grossman, J. and Weissmann, G. (1970) Effect of cyclic 3′,5′-adenosine monophosphate and theophylline on lymphocyte transformation. Proc. Soc. Exp. Biol. Med. **133**, 1361–1365
17 Smith, J. W., Steiner, A. L. and Parker, C. W. (1971) Human lymphocytic metabolism: effects of cyclic and noncyclic nucleotides on stimulation by phytohemagglutinin. J. Clin. Invest. **50**, 442–446
18 Epstein, P. M., Mills, J. S., Harsh, E. M., Strada, S. J. and Thompson, W. J. (1980) Activation of cyclic nucleotide phosphodiesterase from isolated human peripheral blood lymphocytes by mitogenic agents. Cancer Res. **40**, 379–386
19 Epstein, P. M., Mills, J. S., Ross, C. P., Strada, S. J., Hersh, E. M. and Thompson, W. J. (1977) Increased cyclic nucleotide phosphodiesterase activity associated with proliferation and cancer in human and murine lymphoid cells. Cancer Res. **37**, 4016–4023
20 Hait, W. N. and Weiss, B. (1976) Increased cyclic nucleotide phosphodiesterase activity in leukaemic lymphocytes. Nature (London) **259**, 321–323
21 Epstein, P. M. and Hachisu, R. (1984) Cyclic nucleotide phosphodiesterase in normal and leukemic human lymphocytes and lymphoblasts. Adv. Cyclic Nucleotide Protein Phosphorylation Res. **16**, 303–324
22 Houslay, M. D. and Milligan, G. (1997) Tailoring cAMP-signalling responses through isoform multiplicity. Trends Biochem. Sci. **22**, 217–224
23 Beavo, J. A. and Brunton, L. L. (2002) Cyclic nucleotide research – still expanding after half a century. Nat. Rev. Mol. Cell Biol. **3**, 710–718
24 Houslay, M. D. (1998) Adaptation in cyclic AMP signalling processes: a central role for cyclic AMP phosphodiesterases. Semin. Cell Dev. Biol. **9**, 161–167
25 Rich, T. C., Tse, T. E., Rohan, J. G., Schaack, J. and Karpen, J. W. (2001) *In vivo* assessment of local phosphodiesterase activity using tailored cyclic nucleotide-gated channels as cAMP sensors. J. Gen. Physiol. **118**, 63–78
26 Nikolaev, V. O., Gambaryan, S., Engelhardt, S., Walter, U. and Lohse, M. J. (2005) Real-time monitoring of the PDE2 activity of live cells: hormone-stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis. J. Biol. Chem. **280**, 1716–1719
27 Soderling, S. H. and Beavo, J. A. (2000) Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. Curr. Opin. Cell Biol. **12**, 174–179
28 Francis, S. H., Turko, I. V. and Corbin, J. D. (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. Prog. Nucleic Acid Res. Mol. Biol. **65**, 1–52
29 Castro, A., Jerez, M. J., Gil, C. and Martinez, A. (2005) Cyclic nucleotide phosphodiesterases and their role in immunomodulatory responses: advances in the development of specific phosphodiesterase inhibitors. Med. Res. Rev. **25**, 229–244
30 Burnouf, C. and Pruniaux, M. P. (2002) Recent advances in PDE4 inhibitors as immunoregulators and anti-inflammatory drugs. Curr. Pharm. Des. **8**, 1255–1296
31 Essayan, D. M. (2001) Cyclic nucleotide phosphodiesterases. J. Allergy Clin. Immunol. **108**, 671–680
32 Souness, J. E., Aldous, D. and Sargent, C. (2000) Immunosuppressive and anti-inflammatory effects of cyclic AMP phosphodiesterase (PDE) type 4 inhibitors. Immunopharmacology **47**, 127–162
33 Hirsh, L., Dantes, A., Suh, B. S., Yoshida, Y., Hosokawa, K., Tajima, K., Kotsuji, F., Merimsky, O. and Amsterdam, A. (2004) Phosphodiesterase inhibitors as anti-cancer drugs. Biochem. Pharmacol. **68**, 987–988
34 Marko, D., Pahlke, G., Merz, K. H. and Eisenbrand, G. (2000) Cyclic 3′,5′-nucleotide phosphodiesterases: potential targets for anticancer therapy. Chem. Res. Toxicol. **13**, 944–948
35 Lemer, A., Kim, D. H. and Lee, R. (2000) The cAMP signaling pathway as a therapeutic target in lymphoid malignancies. Leuk. Lymphoma **37**, 39–51
36 Kakkar, R., Raju, R. V. and Sharma, R. K. (1999) Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1). Cell. Mol. Life Sci. **55**, 1164–1186
37 Goraya, T. A. and Cooper, D. M. (2005) Ca^{2+}-calmodulin-dependent phosphodiesterase (PDE1): current perspectives. Cell. Signalling **17**, 789–797
38 Zhao, A. Z., Yan, C., Sonnenburg, W. K. and Beavo, J. A. (1997) Recent advances in the study of Ca^{2+}/CaM-activated phosphodiesterases: expression and physiological functions. Adv. Second Messenger Phosphoprotein Res. **31**, 237–251
39 Hagiwara, M., Endo, T. and Hidaka, H. (1984) Effects of vinpocetine on cyclic nucleotide metabolism in vascular smooth muscle. Biochem. Pharmacol. **33**, 453–457
40 Lorenz, K. L. and Wells, J. N. (1983) Potentiation of the effects of sodium nitroprusside and of isoproterenol by selective phosphodiesterase inhibitors. Mol. Pharmacol. **23**, 424–430
41 Ahn, H. S., Crim, W., Romano, M., Sybertz, E. and Pitts, B. (1989) Effects of selective inhibitors on cyclic nucleotide phosphodiesterases of rabbit aorta. Biochem. Pharmacol. **38**, 3331–3339
42 Han, P., Werber, J., Surana, M., Fleischer, N. and Michaeli, T. (1999) The calcium/calmodulin-dependent phosphodiesterase PDE1C down-regulates glucose-induced insulin secretion. J. Biol. Chem. **274**, 22337–22344
43 Sitges, M. and Nekrassov, V. (1999) Vinpocetine selectively inhibits neurotransmitter release triggered by sodium channel activation. Neurochem. Res. **24**, 1585–1591
44 Vemulapalli, S., Watkins, R. W., Chintala, M., Davis, H., Ahn, H. S., Fawzi, A., Tulshian, D., Chiu, P., Chatterjee, M., Lin, C. C. and Sybertz, E. J. (1996) Antiplatelet and antiproliferative effects of SCH 51866, a novel type 1 and type 5 phosphodiesterase inhibitor. J. Cardiovasc. Pharmacol. **28**, 862–869
45 Ahn, H. S., Crim, W., Pitts, B. and Sybertz, E. J. (1992) Calcium-calmodulin-stimulated and cyclic-GMP-specific phosphodiesterases: tissue distribution, drug sensitivity, and regulation of cyclic GMP levels. Adv. Second Messenger Phosphoprotein Res. **25**, 271–288
46 Levin, R. M. and Weiss, B. (1979) Selective binding of antipsychotics and other psychoactive agents to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. J. Pharmacol. Exp. Ther. **208**, 454–459
47 Volpi, M., Shafaei, R. I., Epstein, P. M., Andrenyak, D. M. and Feinstein, M. B. (1981) Local anesthetics, mepacrine, and propranolol are antagonists of calmodulin. Proc. Natl. Acad. Sci. U.S.A. **78**, 795–799
48 Cheng, K. C., Lambert, J. J., Henderson, E. G., Smilowitz, H. and Epstein, P. M. (1981) Postsynaptic inhibition of neuromuscular transmission by trifluoperazine. J. Pharmacol. Exp. Ther. **217**, 44–50
49 Jiang, X., Li, J., Paskind, M. and Epstein, P. M. (1996) Inhibition of calmodulin-dependent phosphodiesterase induces apoptosis in human leukemic cells. Proc. Natl. Acad. Sci. U.S.A. **93**, 11236–11241
50 Epstein, P. M. (1996) Antisense inhibition of phosphodiesterase expression. Methods **14**, 21–33
51 Medema, R. H. (2004) Optimizing RNA interference for application in mammalian cells. Biochem. J. **380**, 593–603
52 Lynch, M. J., Baillie, G. S., Mohamed, A., Li, X., Maisonneuve, C., Klussmann, E., van Heeke, G. and Houslay, M. D. (2005) RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with β-arrestin to control the PKA/AKAP79-mediated switching of the β_2-adrenergic receptor to activation of ERK in HEK293 cells. J. Biol. Chem. **280**, 33178–33189
53 Kanda, N. and Watanabe, S. (2001) Regulatory roles of adenylate cyclase and cyclic nucleotide phosphodiesterases 1 and 4 in interleukin-13 production by activated human T cells. Biochem. Pharmacol. **62**, 495–507
54 Gantner, F., Gotz, C., Geketer, V., Schudt, C., Wendel, A. and Hatzelmann, A. (1998) Phosphodiesterase profile of human B lymphocytes from normal and atopic donors and the effects of PDE inhibition on B cell proliferation. Br. J. Pharmacol. **123**, 1031–1038
55 Epstein, P. M., Moraski, Jr, S. and Hachisu, R. (1987) Identification and characterization of a Ca^{2+}-calmodulin-sensitive cyclic nucleotide phosphodiesterase in a human lymphoblastoid cell line. Biochem. J. **243**, 533–539
56 Gantner, F., Schudt, C., Wendel, A. and Hatzelmann, A. (1999) Characterization of the phosphodiesterase (PDE) pattern of *in vitro*-generated human dendritic cells (DC) and the influence of PDE inhibitors on DC function. Pulm. Pharmacol. Ther. **12**, 377–386
57 Bender, A. T., Ostenson, C. L., Wang, E. H. and Beavo, J. A. (2005) Selective up-regulation of PDE1B2 upon monocyte-to-macrophage differentiation. Proc. Natl. Acad. Sci. U.S.A. **102**, 497–502
58 Reed, T. M., Repaske, D. R., Snyder, G. L., Greengard, P. and Vorhees, C. V. (2002) Phosphodiesterase 1B knock-out mice exhibit exaggerated locomotor hyperactivity and DARPP-32 phosphorylation in response to dopamine agonists and display impaired spatial learning. J. Neurosci. **22**, 5188–5197
59 Martins, T. J., Mumby, M. C. and Beavo, J. A. (1982) Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. J. Biol. Chem. **257**, 1973–1979
60 Ho, Y. S., Burden, L. M. and Hurley, J. H. (2000) Structure of the GAF domain, a ubiquitous signaling motif and a new class of cyclic GMP receptor. EMBO J. **19**, 5288–5299
61 Martinez, S. E., Beavo, J. A. and Hol, W. G. (2002) GAF domains: two-billion-year-old molecular switches that bind cyclic nucleotides. Mol. Interventions **2**, 317–323
62 Zoraghi, R., Corbin, J. D. and Francis, S. H. (2004) Properties and functions of GAF domains in cyclic nucleotide phosphodiesterases and other proteins. Mol. Pharmacol. **65**, 267–278

63 Martinez, S. E., Wu, A. Y., Glavas, N. A., Tang, X. B., Turley, S., Hol, W. G. and Beavo, J. A. (2002) The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and in cGMP binding. Proc. Natl. Acad. Sci. U.S.A. 99, 13260–13265
64 Sonnenburg, W. K., Mullaney, P. J. and Beavo, J. A. (1991) Molecular cloning of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase cDNA: identification and distribution of isozyme variants. J. Biol. Chem. 266, 17655–17661
65 Yang, Q., Paskind, M., Bolger, G., Thompson, W. J., Repaske, D. R., Cutler, L. S. and Epstein, P. M. (1994) A novel cyclic GMP stimulated phosphodiesterase from rat brain. Biochem. Biophys. Res. Commun. 205, 1850–1856
66 Rosman, G. J., Martins, T. J., Sonnenburg, W. K., Beavo, J. A., Ferguson, K. and Loughney, K. (1997) Isolation and characterization of human cDNAs encoding a cGMP-stimulated 3′,5′-cyclic nucleotide phosphodiesterase. Gene 191, 89–95
67 Epstein, P. M., Paskind, M., Yang, Q., Cong, H., Chen, J. C., Li, J. and Reenan, R. (1998) Co-expression of three splice variants of cGMP-stimulated phosphodiesterase (PDE2) in rat brain. Naunyn Schmiedeberg's Arch. Pharmacol. 358, R661
68 Podzuweit, T., Nennstiel, P. and Muller, A. (1995) Isozyme selective inhibition of cGMP-stimulated cyclic nucleotide phosphodiesterases by erythro-9-(2-hydroxy-3-nonyl) adenine. Cell. Signalling 7, 733–738
69 Mery, P. F., Pavoine, C., Pecker, F. and Fischmeister, R. (1995) Erythro-9-(2-hydroxy-3-nonyl)adenine inhibits cyclic GMP-stimulated phosphodiesterase in isolated cardiac myocytes. Mol. Pharmacol. 48, 121–130
70 Boess, F. G., Hendrix, M., van der Staay, F. J., Erb, C., Schreiber, R., van Staveren, W., de Vente, J., Prickaerts, J., Blokland, A. and Koenig, G. (2004) Inhibition of phosphodiesterase 2 increases neuronal cGMP, synaptic plasticity and memory performance. Neuropharmacology 47, 1081–1092
71 Netherton, S. J. and Maurice, D. H. (2005) Vascular endothelial cell cyclic nucleotide phosphodiesterases and regulated cell migration: implications in angiogenesis. Mol. Pharmacol. 67, 253–272
72 Dickinson, N. T., Jang, E. K. and Haslam, R. J. (1997) Activation of cGMP-stimulated phosphodiesterase by nitroprusside limits cAMP accumulation in human platelets: effects on platelet aggregation. Biochem. J. 323, 371–377
73 Manns, J. M., Brenna, K. J., Colman, R. W. and Sheth, S. B. (2002) Differential regulation of human platelet responses by cGMP inhibited and stimulated cAMP phosphodiesterases. Thromb. Haemostasis 87, 873–879
74 Michie, A. M., Lobban, M., Muller, T., Harnett, M. M. and Houslay, M. D. (1996) Rapid regulation of PDE-2 and PDE-4 cyclic AMP phosphodiesterase activity following ligation of the T cell antigen receptor on thymocytes: analysis using the selective inhibitors erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) and rolipram. Cell. Signalling 8, 97–110
75 Tenor, H., Staniciu, L., Schudt, C., Hatzelmann, A., Wendel, A., Djukanovic, R., Church, M. K. and Shute, J. K. (1995) Cyclic nucleotide phosphodiesterases from purified human CD4$^+$ and CD8$^+$ T lymphocytes. Clin. Exp. Allergy 25, 616–624
76 Bender, A. T., Ostenson, C. L., Giordano, D. and Beavo, J. A. (2004) Differentiation of human monocytes *in vitro* with granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor produces distinct changes in cGMP phosphodiesterase expression. Cell. Signalling 16, 365–374
77 Degerman, E., Belfrage, P. and Manganiello, V. C. (1996) cGMP-inhibited phosphodiesterases (PDE3 gene family). Biochem. Soc. Trans. 24, 1010–1014
78 Shakur, Y., Holst, L. S., Landstrom, T. R., Movsesian, M., Degerman, E. and Manganiello, V. (2001) Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family. Prog. Nucleic Acid Res. Mol. Biol. 66, 241–277
79 Movsesian, M. A. (2002) PDE3 cyclic nucleotide phosphodiesterases and the compartmentation of cyclic nucleotide-mediated signalling in cardiac myocytes. Basic Res. Cardiol. 97 (suppl. 1), 83–90
80 Kambayashi, J., Liu, Y., Sun, B., Shakur, Y., Yoshitake, M. and Czerwiec, F. (2003) Cilostazol as a unique antithrombotic agent. Curr. Pharm. Des. 9, 2289–2302
81 Shakur, Y., Fong, M., Hensley, J., Cone, J., Movsesian, M. A., Kambayashi, J., Yoshitake, M. and Liu, Y. (2002) Comparison of the effects of cilostazol and milrinone on cAMP-PDE activity, intracellular cAMP and calcium in the heart. Cardiovasc. Drugs Ther. 16, 417–427
82 Edmondson, S. D., Mastracchio, A., He, J., Chung, C. C., Forrest, M. J., Holsess, S., MacIntyre, E., Metzger, J., O'Connor, N., Patel, K. et al. (2003) Benzyl vinylogous amide substituted aryldihydropyridazinones and aryldimethylpyrazolones as potent and selective PDE3B inhibitors. Bioorg. Med. Chem. Lett. 13, 3985–3987
83 Feilge, M. A., Ansink, K., Vanschoonbeek, K. and Heemskerk, J. W. (2004) Control of platelet activation by cyclic AMP turnover and cyclic nucleotide phosphodiesterase type-3. Biochem. Pharmacol. 67, 1559–1567
84 Jensen, B. O., Selheim, F., Doskeland, S. O., Gear, A. R. and Holmsen, H. (2004) Protein kinase A mediates inhibition of the thrombin-induced platelet shape change by nitric oxide. Blood 104, 2775–2782
85 Masciarelli, S., Horner, K., Liu, C., Park, S. H., Hinckley, M., Hockman, S., Nedachi, T., Jin, C., Conti, M. and Manganiello, V. (2004) Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. J. Clin. Invest. 114, 196–205
86 Sheth, S. B., Chaganti, K., Bastepe, M., Ajuria, J., Brennan, K., Giradavolu, R. and Colman, R. W. (1997) Cyclic AMP phosphodiesterases in human lymphocytes. Br. J. Haematol. 99, 784–789
87 Seybold, J., Newton, R., Wright, L., Finney, P. A., Sultorp, N., Barnes, P. J., Adcock, I. M. and Giembycz, M. A. (1998) Induction of phosphodiesterases 3B, 4A4, 4D1, 4D2, and 4D3 in Jurkat T-cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and G_s-coupled receptor agonists. Potential role in β_2-adrenoreceptor desensitization. J. Biol. Chem. 273, 20575–20588
88 Ercegar, S. and Houslay, M. D. (1997) Challenge of human Jurkat T-cells with the adenylate cyclase activator forskolin elicits major changes in cAMP phosphodiesterase (PDE) expression by up-regulating PDE3 and inducing PDE4D1 and PDE4D2 splice variants as well as down-regulating a novel PDE4A splice variant. Biochem. J. 321, 165–175
89 Bielekova, B., Lincoln, A., McFarland, H. and Martin, R. (2000) Therapeutic potential of phosphodiesterase-4 and -3 inhibitors in Th1-mediated autoimmune diseases. J. Immunol. 164, 1117–1124
90 Dousa, M. K., Moore, S. B., Ploeger, N. A., DeGoey, S. R. and Dousa, T. P. (1997) Antagonists of cyclic nucleotide phosphodiesterase (PDE) isozymes PDE 3 and PDE 4 suppress lymphoblastic response to HLA class II alloantigens: a potential novel approach to preventing allograft rejection? Clin. Nephrol. 47, 187–189
91 Giembycz, M. A., Corrigan, C. J., Seybold, J., Newton, R. and Barnes, P. J. (1996) Identification of cyclic AMP phosphodiesterases 3, 4 and 7 in human CD4$^+$ and CD8$^+$ T-lymphocytes: role in regulating proliferation and the biosynthesis of interleukin-2. Br. J. Pharmacol. 118, 1945–1958
92 Schudt, C., Tenor, H. and Hatzelmann, A. (1995) PDE isoenzymes as targets for anti-asthma drugs. Eur. Respir. J. 8, 1179–1183
93 Robicsek, S. A., Blanchard, D. K., Djeu, J. Y., Krzanowski, J. J., Szentivanyi, A. and Polson, J. B. (1991) Multiple high-affinity cAMP-phosphodiesterases in human T-lymphocytes. Biochem. Pharmacol. 42, 869–877
94 Schudt, C., Winder, S., Eltze, M., Killian, U. and Beume, R. (1991) Zardaverine: a cyclic AMP specific PDE III/IV inhibitor. Agents Actions Suppl. 34, 379–402
95 Giembycz, M. A. (2005) Life after PDE4: overcoming adverse events with dual-specificity phosphodiesterase inhibitors. Curr. Opin. Pharmacol. 5, 238–244
96 Moon, E., Lee, R., Near, R., Weintraub, L., Wolda, S. and Lerner, A. (2002) Inhibition of PDE3B augments PDE4 inhibitor-induced apoptosis in a subset of patients with chronic lymphocytic leukemia. Clin. Cancer Res. 8, 589–595
97 Inoue, A., Kuroyanagi, Y., Terui, K., Mol, P. and Ikuta, T. (2004) Negative regulation of γ-globin gene expression by cyclic AMP-dependent pathway in erythroid cells Exp. Hematol. 32, 244–253
98 Ahmad, F., Gao, G., Wang, L. M., Landstrom, T. R., Degerman, E., Pierce, J. H. and Manganiello, V. C. (1999) IL-3 and IL-4 activate cyclic nucleotide phosphodiesterases 3 (PDE3) and 4 (PDE4) by different mechanisms in FDCP2 myeloid cells. J. Immunol. 162, 4864–4875
99 Ahmad, F., Cong, L. N., Stenson Holst, L., Wang, L. M., Rahn Landstrom, T., Pierce, J. H., Quon, M. J., Degerman, E. and Manganiello, V. C. (2000) Cyclic nucleotide phosphodiesterase 3B is a downstream target of protein kinase B and may be involved in regulation of effects of protein kinase B on thymidine incorporation in FDCP2 cells. J. Immunol. 164, 4678–4688
100 Zhao, A. Z., Huan, J. N., Gupta, S., Pal, R. and Sahu, A. (2002) A phosphatidylinositol 3-kinase phosphodiesterase 3B-cyclic AMP pathway in hypothalamic action of leptin on feeding. Nat. Neurosci. 5, 727–728
101 Landells, L. J., Spina, D., Souness, J. E., O'Connor, B. J. and Page, C. P. (2000) A biochemical and functional assessment of monocyte phosphodiesterase activity in healthy and asthmatic subjects. Pulm. Pharmacol. Ther. 13, 231–239
102 Gantner, F., Tenor, H., Gekeler, V., Schudt, C., Wendel, A. and Hatzelmann, A. (1997) Phosphodiesterase profiles of highly purified human peripheral blood leucocyte populations from normal and atopic individuals: a comparative study. J. Allergy Clin. Immunol. 100, 527–535
103 Gantner, F., Kupferschmidt, R., Schudt, C., Wendel, A. and Hatzelmann, A. (1997) *In vitro* differentiation of human monocytes to macrophages: change of PDE profile and its relationship to suppression of tumour necrosis factor-α release by PDE inhibitors. Br. J. Pharmacol. 121, 221–231
104 Germain, N., Bertin, B., Legendre, A., Martin, B., Lagente, V., Payne, A. and Boichot, E. (1998) Selective phosphodiesterase inhibitors modulate the activity of alveolar macrophages from sensitized guinea-pigs. Eur. Respir. J. 12, 1334–1339
105 Johnston, L. A., Erdogan, S., Cheung, Y. F., Sullivan, M., Barber, R., Lynch, M. J., Baillie, G. S., Van Heeke, G., Adams, D. R., Huston, E. and Houslay, M. D. (2004) Expression, intracellular distribution and basis for lack of catalytic activity of the PDE4A7 isoform encoded by the human PDE4A cAMP-specific phosphodiesterase gene. Biochem. J. 380, 371–384
106 Houslay, M. D. and Adams, D. R. (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. Biochem. J. 370, 1–18

107 Richter, W. and Conti, M. (2002) Dimerization of the type 4 cAMP-specific phosphodiesterases is mediated by the upstream conserved regions (UCRs). J. Biol. Chem. **277**, 40212–40221

108 Jin, S. L., Bushnik, T., Lan, L. and Conti, M. (1998) Subcellular localization of rolipram-sensitive, cAMP-specific phosphodiesterases: differential targeting and activation of the splicing variants derived from the PDE4D gene. J. Biol. Chem. **273**, 19672–19678

109 Bolger, G. B., McCahill, A., Huston, E., Cheung, Y. F., McSorley, T., Baillie, G. S. and Houslay, M. D. (2003) The unique amino-terminal region of the PDE4D5 cAMP phosphodiesterase isoform confers preferential interaction with β-arrestins. J. Biol. Chem. **278**, 49230–49238

110 Bolger, G. B., Peden, A. H., Steele, M. R., MacKenzie, C., McEwan, D. G., Wallace, D. A., Huston, E., Baillie, G. S. and Houslay, M. D. (2003) Attenuation of the activity of the cAMP-specific phosphodiesterase PDE4A5 by interaction with the immunophilin XAP2. J. Biol. Chem. **278**, 33351–33363

111 Dodge, K. L., Khouangsathiene, S., Kapiloff, M. S., Mouton, R., Hill, E. V., Houslay, M. D., Langeberg, L. K. and Scott, J. D. (2001) mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. EMBO J. **20**, 1921–1930

112 Jin, S. L., Richard, F. J., Kuo, W. P., D'Ercole, A. J. and Conti, M. (1999) Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D-deficient mice. Proc. Natl. Acad. Sci. U.S.A. **96**, 11998–12003

113 Hansen, G., Jin, S., Umetsu, D. T. and Conti, M. (2000) Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D. Proc. Natl. Acad. Sci. U.S.A. **97**, 6751–6756

114 Mehats, C., Jin, S. L., Wahlstrom, J., Law, E., Umetsu, D. T. and Conti, M. (2003) PDE4D plays a critical role in the control of airway smooth muscle contraction. FASEB J. **17**, 1831–1841

115 Jin, S. L. and Conti, M. (2002) Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF-α responses. Proc. Natl. Acad. Sci. U.S.A. **99**, 7628–7633

116 Jin, S. L., Lan, L., Zoudilova, M. and Conti, M. (2005) Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages. J. Immunol. **175**, 1523–1531

117 Ariga, M., Neitzert, B., Nakae, S., Mottin, G., Bertrand, C., Pruniaux, M. P., Jin, S. L. and Conti, M. (2004) Nonredundant function of phosphodiesterases 4D and 4B in neutrophil recruitment to the site of inflammation. J. Immunol. **173**, 7531–7538

118 Casacchia, M., Meco, G., Castellana, F., Bedini, L., Cusimano, G. and Agnoli, A. (1983) Therapeutic use of a selective cAMP phosphodiesterase inhibitor (Rolipram) in Parkinson's disease. Pharmacol. Res. Commun. **15**, 329–334

119 Hebenstreit, G. F., Fellerer, K., Fichte, K., Fischer, G., Geyer, N., Meya, U., Sastre-y-Hernandez, M., Schony, W., Schratzer, M., Soukop, W. et al. (1989) Rolipram in major depressive disorder: results of a double-blind comparative study with imipramine. Pharmacopsychiatry **22**, 156–160

120 Robichaud, A., Stamatiou, P. B., Jin, S. L., Lachance, N., MacDonald, D., Laliberte, F., Liu, S., Huang, Z., Conti, M. and Chan, C. C. (2002) Deletion of phosphodiesterase 4D in mice shortens α_2-adrenoceptor-mediated anesthesia, a behavioral correlate of emesis. J. Clin. Invest. **110**, 1045–1052

121 Bundschuh, D. S., Eltze, M., Barsig, J., Wollin, L., Hatzelmann, A. and Beume, R. (2001) *In vivo* efficacy in airway disease models of roflumilast, a novel orally active PDE4 inhibitor. J. Pharmacol. Exp. Ther. **297**, 280–290

122 Timmer, W., Leclerc, V., Birraux, G., Neuhauser, M., Hatzelmann, A., Bethke, T. and Wurst, W. (2002) The new phosphodiesterase 4 inhibitor roflumilast is efficacious in exercise-induced asthma and leads to suppression of LPS-stimulated TNF-α *ex vivo*. J. Clin. Pharmacol. **42**, 297–303

123 Lipworth, B. J. (2005) Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease. Lancet **365**, 167–175

124 Card, G. L., Blasdel, L., England, B. P., Zhang, C., Suzuki, Y., Gillette, S., Fong, D., Ibrahim, P. N., Artis, D. R., Bollag, G. et al. (2005) A family of phosphodiesterase inhibitors discovered by cocrystallography and scaffold-based drug design. Nat. Biotechnol. **23**, 201–207

125 Smith, P. G., Wang, F., Wilkinson, K. N., Savage, K. J., Klein, U., Neuberg, D. S., Bollag, G., Shipp, M. A. and Aguiar, R. C. (2005) The phosphodiesterase PDE4B limits cAMP-associated PI3K/AKT-dependent apoptosis in diffuse large B-cell lymphoma. Blood **105**, 308–316

126 Abrahamsen, H., Baillie, G., Ngai, J., Vang, T., Nika, K., Ruppelt, A., Mustelin, T., Zaccolo, M., Houslay, M. and Tasken, K. (2004) TCR- and CD28-mediated recruitment of phosphodiesterase 4 to lipid rafts potentiates TCR signaling. J. Immunol. **173**, 4847–4858

127 Baillie, G. S., Scott, J. D. and Houslay, M. D. (2005) Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. FEBS Lett. **579**, 3264–3270

128 Brunton, L. L. (2003) PDE4: arrested at the border. Science STKE **2003**, PE44

129 Houslay, M. D. (2001) PDE4 cAMP-specific phosphodiesterases. Prog. Nucleic Acid Res. Mol. Biol. **69**, 249–315

130 Houslay, M. D., Sullivan, M. and Bolger, G. B. (1998) The multienzyme PDE4 cyclic adenosine monophosphate-specific phosphodiesterase family: intracellular targeting, regulation, and selective inhibition by compounds exerting anti-inflammatory and antidepressant actions. Adv. Pharmacol. **44**, 225–342

131 Arp, J., Kirchhof, M. G., Baroja, M. L., Nazarian, S. H., Chau, T. A., Strathdee, C. A., Ball, E. H. and Madrenas, J. (2003) Regulation of T-cell activation by phosphodiesterase 4B2 requires its dynamic redistribution during immunological synapse formation. Mol. Cell. Biol. **23**, 8042–8057

132 Baroja, M. L., Cieslinski, L. B., Torphy, T. J., Wange, R. L. and Madrenas, J. (1999) Specific CD3ϵ association of a phosphodiesterase 4B isoform determines its selective tyrosine phosphorylation after CD3 ligation. J. Immunol. **162**, 2016–2023

133 Jiang, X., Paskind, M., Weltzien, R. and Epstein, P. M. (1998) Expression and regulation of mRNA for distinct isoforms of cAMP-specific PDE-4 in mitogen-stimulated and leukemic human lymphocytes. Cell Biochem. Biophys. **28**, 135–160

134 Tiwari, S., Cong, H., Kim, E. J., Weintraub, L., Epstein, P. M. and Lerner, A. (2005) Type 4 cAMP phosphodiesterase (PDE4) inhibitors augment glucocorticoid-mediated apoptosis in B cell chronic lymphocytic leukemia (B-CLL) in the absence of exogenous adenylyl cyclase stimulation. Biochem. Pharmacol. **69**, 473–483

135 Hidi, R., Timmermans, S., Liu, E., Schuct, C., Dent, G., Holgate, S. T. and Djukanovic, R. (2000) Phosphodiesterase and cyclic adenosine monophosphate-dependent inhibition of T-lymphocyte chemotaxis. Eur. Respir. J. **15**, 342–349

136 Layseca-Espinosa, E., Baranda, L., Alvarado-Sanchez, B., Portales-Perez, D., Portillo-Salazar, H. and Gonzalez-Amaro, R. (2003) Rolipram inhibits polarization and migration of human T lymphocytes. J. Invest. Dermatol. **121**, 81–87

137 Sommer, N., Martin, R., McFarland, H. F., Quigley, L., Cannella, B., Raine, C. S., Scott, D. E., Loschmann, P. A. and Racke, M. K. (1997) Therapeutic potential of phosphodiesterase type 4 inhibition in chronic autoimmune demyelinating disease. J. Neuroimmunol. **79**, 54–61

138 Shepherd, M. C., Baillie, G. S., Stirling, D. I. and Houslay, M. D. (2004) Remodelling of the PDE4 cAMP phosphodiesterase isoform profile upon monocyte–macrophage differentiation of human U937 cells. Br. J. Pharmacol. **142**, 339–351

139 Barber, R., Baillie, G. S., Bergmann, R., Shepherd, M. C., Seppet, R., Houslay, M. D. and Heeke, G. V. (2004) Differential expression of PDE4 cAMP phosphodiesterase isoforms in inflammatory cells of smokers with COPD, smokers without COPD, and nonsmokers. Am. J. Physiol. Lung Cell. Mol. Physiol. **287**, L332–L343

140 Heystek, H. C., Thierry, A. C., Soulard, P. and Moulon, C. (2003) Phosphodiesterase 4 inhibitors reduce human dendritic cell inflammatory cytokine production and Th1-polarizing capacity. Int. Immunol. **15**, 827–835

141 Wang, P., Wu, P., Ohleth, K. M., Egan, R. W. and Billah, M. M. (1999) Phosphodiesterase 4B2 is the predominant phosphodiesterase species and undergoes differential regulation of gene expression in human monocytes and neutrophils. Mol. Pharmacol. **56**, 170–174

142 Jones, N. A., Boswell-Smith, V., Lever, R. and Page, C. P. (2005) The effect of selective phosphodiesterase isoenzyme inhibition on neutrophil function *in vitro*. Pulm. Pharmacol. Ther. **18**, 93–101

143 Jacob, C., Szilagyi, C., Allen, J. M., Bertrand, C. and Lagente, V. (2004) Role of PDE4 in superoxide anion generation through p44/42^{MAPK} regulation: a cAMP and a PKA-independent mechanism. Br. J. Pharmacol. **143**, 257–268

144 Au, B. T., Teixeira, M. M., Collins, P. D. and Williams, T. J. (1998) Effect of PDE4 inhibitors on zymosan-induced IL-8 release from human neutrophils: synergism with prostanoids and salbutamol. Br. J. Pharmacol. **123**, 1260–1266

145 Barnette, M. S., Christensen, S. B., Essayan, D. M., Grous, M., Prabhakar, U., Rush, J. A., Kagey-Sobotka, A. and Torphy, T. J. (1998) SB 207499 (Ariflo), a potent and selective second-generation phosphodiesterase 4 inhibitor: *in vitro* anti-inflammatory actions. J. Pharmacol. Exp. Ther. **284**, 420–426

146 Wallis, R. M., Corbin, J. D., Francis, S. H. and Ellis, P. (1999) Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings *in vitro*. Am. J. Cardiol. **83**, 3C–12C

147 Toward, T. J., Smith, N. and Broadley, K. J. (2004) Effect of phosphodiesterase-5 inhibitor, sildenafil (Viagra), in animal models of airways disease. Am. J. Respir. Crit. Care Med. **169**, 227–234

148 Zhang, X. and Cote, R. H. (2005) cGMP signaling in vertebrate retinal photoreceptor cells. Front. Biosci. **10**, 1191–1204

149 Wang, H., Lee, Y. and Malbon, C. C. (2004) PDE6 is an effector for the Wnt/Ca^{2+}/cGMP-signalling pathway in development. Biochem. Soc. Trans. **32**, 792–795

150 Michaeli, T., Bloom, T. J., Martins, T., Loughney, K., Ferguson, K., Riggs, M., Rodgers, L., Beavo, J. A. and Wigler, M. (1993) Isolation and characterization of a previously undetected human cAMP phosphodiesterase by complementation of cAMP phosphodiesterase-deficient *Saccharomyces cerevisiae*. J. Biol. Chem. **268**, 12925–12932

151 Helman, J. M., Soderling, S. H., Glavas, N. A. and Beavo, J. A. (2000) Cloning and characterization of PDE7B, a cAMP-specific phosphodiesterase. Proc. Natl. Acac. Sci. U.S.A. **97**, 472–476
152 Gardner, C., Robas, N., Cawkill, D. and Fidock, M. (2000) Cloning and characterization of the human and mouse PDE7B, a novel cAMP-specific cyclic nucleotide phosphodiesterase. Biochem. Biophys. Res. Commun. **272**, 186–192
153 Ichimura, M. and Kase, H. (1993) A new cyclic nucleotide phosphodiesterase isozyme expressed in the T-lymphocyte cell lines. Biochem. Biophys. Res. Commun. **193**, 985–990
154 Lee, R., Wolda, S., Moon, E., Esselstyn, J., Hertel, C. and Lerner, A. (2002) PDE7A is expressed in human B-lymphocytes and is up-regulated by elevation of intracellular cAMP. Cell. Signalling **14**, 277–284
155 Smith, S. J., Brookes-Fazakerley, S., Donnelly, L. E., Barnes, P. J., Barnette, M. S. and Giembycz, M. A. (2003) Ubiquitous expression of phosphodiesterase 7A in human proinflammatory and immune cells. Am. J. Physiol. Lung Cell. Mol. Physiol. **284**, L279–L289
156 Asirvatham, A. L., Galligan, S. G., Schillace, R. V., Davey, M. P., Vasta, V., Beavo, J. A. and Carr, D. W. (2004) A-kinase anchoring proteins interact with phosphodiesterases in T lymphocyte cell lines. J. Immunol. **173**, 4806–4814
157 Li, L., Yee, C. and Beavo, J. A. (1999) CD3- and CD28-dependent induction of PDE7 required for T cell activation. Science **283**, 848–851
158 Glavas, N. A., Ostenson, C., Schaefer, J. B., Vasta, V. and Beavo, J. A. (2001) T cell activation up-regulates cyclic nucleotide phosphodiesterases 8A1 and 7A3. Proc. Natl. Acad. Sci. U.S.A. **98**, 6319–6324
159 Yang, G., McIntyre, K. W., Townsend, R. M., Shen, H. H., Pitts, W. J., Dodd, J. H., Nadler, S. G., McKlanon, M. and Watson, A. J. (2003) Phosphodiesterase 7A-deficient mice have functional T cells. J. Immunol. **171**, 6414–6420
160 Smith, S. J., Cieslinski, L. B., Newton, R., Donnelly, L. E., Fenwick, P. S., Nicholson, A. G., Barnes, P. J., Barnette, M. S. and Giembycz, M. A. (2004) Discovery of BRL 50481 [3-(*N*,*N*-dimethylsulphonamido)-4-methyl-nitrobenzene], a selective inhibitor of phosphodiesterase 7: *in vitro* studies in human monocytes, lung macrophages, and CD8$^+$ T-lymphocytes. Mol. Pharmacol. **66**, 1679–1689
161 Nakata, A., Ogawa, K., Sasaki, T., Koyama, N., Wada, K., Kotera, J., Kikkawa, H., Omori, K. and Kaminuma, O. (2002) Potential role of phosphodiesterase 7 in human T cell function: comparative effects of two phosphodiesterase inhibitors. Clin. Exp. Immunol. **128**, 460–466
162 Soderling, S. H., Bayuga, S. J. and Beavo, J. A. (1998) Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. Proc. Natl. Acad. Sci. U.S.A. **95**, 8991–8996
163 Fisher, D. A., Smith, J. F., Pillar, J. S., St Denis, S. H. and Cheng, J. B. (1998) Isolation and characterization of PDE8A, a novel human cAMP-specific phosphodiesterase. Biochem. Biophys. Res. Commun. **246**, 570–577
164 Hayashi, M., Matsushima, K., Ohashi, H., Tsunoda, H., Murase, S., Kawarada, Y. and Tanaka, T. (1998) Molecular cloning and characterization of human PDE8B, a novel thyroid-specific isozyme of 3′,5′-cyclic nucleotide phosphodiesterase. Biochem. Biophys. Res. Commun. **250**, 751–756
165 Perez-Torres, S., Cortes, R., Tolnay, M., Probst, A., Palacios, J. M. and Mengod, G. (2003) Alterations on phosphodiesterase type 7 and 8 isozyme mRNA expression in Alzheimer's disease brains examined by in situ hybridization. Exp. Neurol. **182**, 322–334
166 Gamanuma, M., Yuasa, K., Sasaki, T., Sakurai, N., Kotera, J. and Omori, K. (2003) Comparison of enzymatic characterization and gene organization of cyclic nucleotide phosphodiesterase 8 family in humans. Cell. Signalling **15**, 565–574
167 Gu, Y. Z., Hogenesch, J. B. and Bradfield, C. A. (2000) The PAS superfamily: sensors of environmental and developmental signals. Annu. Rev. Pharmacol. Toxicol. **40**, 519–561
168 Kewley, R. J., Whitelaw, M. L. and Chapman-Smith, A. (2004) The mammalian basic helix–loop–helix/PAS family of transcriptional regulators. Int. J. Biochem. Cell Biol. **36**, 189–204
169 Wu, P. and Wang, F. (2004) Per-Arnt-Sim domain-dependent association of cAMP-phosphodiesterase 8A1 with br5 proteins. Proc. Natl. Acad. Sci. U.S.A. **101**, 17634–17639
170 Soderling, S. H., Bayuga, S. J. and Beavo, J. A. (1998) Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. J. Biol. Chem. **273**, 15553–15558
171 Fisher, D. A., Smith, J. F., Pillar, J. S., St Denis, S. H. and Cheng, J. B. (1998) Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. J. Biol. Chem. **273**, 15559–15564
172 Andreeva, S. G., Dikkes, P., Epstein, P. M. and Rosenberg, P. A. (2001) Expression of cGMP-specific phosphodiesterase 9A mRNA in the rat brain. J. Neurosci. **21**, 9068–9076
173 Loughney, K., Snyder, P. B., Uher, L., Rosman, G. J., Ferguson, K. and Florio, V. A. (1999) Isolation and characterization of PDE10A, a novel human 3′,5′-cyclic nucleotide phosphodiesterase. Gene **234**, 109–117
174 Soderling, S. H., Bayuga, S. J. and Beavo, J. A. (1999) Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A. Proc. Natl. Acad. Sci. U.S.A. **96**, 7071–7076
175 Pyne, N. J. and Furman, B. L. (2003) Cyclic nucleotide phosphodiesterases in pancreatic islets. Diabetologia **46**, 1179–1189
176 Seeger, T. F., Bartlett, B., Coskran, T. M., Culp, J. S., James, L. C., Krull, D. L., Lanfear, J., Ryan, A. M., Schmidt, C. J., Strick, C. A. et al. (2003) Immunohistochemical localization of PDE10A in the rat brain. Brain Res. **985**, 113–126
177 Hu, H., McCaw, E. A., Hebb, A. L., Gomez, G. T. and Denovan-Wright, E. M. (2004) Mutant huntingtin affects the rate of transcription of striatum-specific isoforms of phosphodiesterase 10A. Eur. J. Neurosci. **20**, 3351–3363
178 Hebb, A. L., Robertson, H. A. and Denovan-Wright, E. M. (2004) Striatal phosphodiesterase mRNA and protein levels are reduced in Huntingtons disease transgenic mice prior to the onset of motor symptoms. Neuroscience **123**, 967–981
179 Fujishige, K., Kotera, J., Michibata, H., Yuasa, K., Takebayashi, S., Okumura, K. and Omori, K. (1999) Cloning and characterization of a novel human phosphodiesterase that hydrolyzes both cAMP and cGMP (PDE10A). J. Biol. Chem. **274**, 18438–18445
180 Schmidt, C., Chambers, L., Chapin, D., Fox, C., Fujiwara, R., Harms, J., McCarthy, S., Murphy, E. and Siuciak, J. (2002) Effect of PDE10 inhibition on striatal cyclic nucleotide concentration. Soc. Neurosci. Abstr. **43**, 19
181 Kleiman, R. J., Brown, T. M., Collins, M. A., Fox, C. B., Kimmel, L. H., Menniti, F., Schmidt, C. J., Shrikhande, A. and Wong, S. (2002) PDE10 Regulation of intracellular signaling in striatal medium spiny neurons in culture. Soc. Neurosci. Abstr. **43**, 18
182 Rodefer, J. S., Murphy, E. R. and Baxter, M. G. (2005) PDE10A inhibition reverses subchronic PCP-induced deficits in attentional set-shifting in rats. Eur. J. Neurosci. **21**, 1070–1076
183 Yuasa, K., Kotera, J., Fujishige, K., Michibata, H., Sasaki, T. and Omori, K. (2000) Isolation and characterization of two novel phosphodiesterase PDE11A variants showing unique structure and tissue-specific expression. J. Biol. Chem. **275**, 31469–31479
184 Fawcett, L., Baxendale, R., Stacey, P., McGrouther, C., Harrow, I., Soderling, S., Hetman, J., Beavo, J. A. and Phillips, S. C. (2000) Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. Proc. Natl. Acad. Sci. U.S.A. **97**, 3702–3707
185 Hetman, J. M., Robas, N., Baxendale, R., Fidock, M., Phillips, S. C., Soderling, S. H. and Beavo, J. A. (2000) Cloning and characterization of two splice variants of human phosphodiesterase 11A. Proc. Natl. Acad. Sci. U.S.A. **97**, 12891–12895
186 Wayman, C., Phillips, S., Lumley, C., Webb, T., Fawcett, L., Baxendale, R. and Burgess, G. (2005) Phosphodiesterase 11 (PDE11) regulation of spermatozoa physiology. Int. J. Impotence Res. **17**, 216–223
187 Loughney, K., Taylor, J. and Florio, V. A. (2005) 3′,5′-Cyclic nucleotide phosphodiesterase 11A: localization in human tissues. Int. J. Impotence Res. **17**, 320–325
188 Weeks, J. L., Zoraghi, R., Beasley, A., Sekhar, K. R., Francis, S. H. and Corbin, J. D. (2005) High biochemical selectivity of tadalafil, sildenafil and vardenafil for human phosphodiesterase 5A1 (PDE5) over PDE11A4 suggests the absence of PDE11A4 cross-reaction in patients. Int. J. Impotence Res. **17**, 5–9
189 Hoffman, A., Cheok, M. H., den Boer, M. L., Yang, W., Veerman, A. J., Kazemier, K. M., Pei, D., Cheng, C., Pui, C. H., Relling, M. V. et al. (2004) Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N. Engl. J. Med. **351**, 533–542
190 Ploner, C., Schmidt, S., Presul, E., Renner, K., Schrocksnadel, K., Rainer, J., Riml, S. and Kofler, R. (2005) Glucocorticoid-induced apoptosis and glucocorticoid resistance in acute lymphoblastic leukemia. J. Steroid Biochem. Mol. Biol. **93**, 153–160
191 Frankfurt, O. and Rosen, S. T. (2004) Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates. Curr. Opin. Oncol. **16**, 553–563
192 Schmidt, S., Rainer, J., Ploner, C., Presul, E., Riml, S. and Kofler, R. (2004) Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell. Death Differ. **11** (suppl. 1), S45–S55
193 Haarman, E. G., Kaspers, G. J. and Veerman, A. J. (2003) Glucocorticoid resistance in childhood leukaemia: mechanisms and modulation. Br. J. Haematol. **120**, 919–929
194 Tissing, W. J., Meijerink, J. P., den Boer, M. L. and Pieters, R. (2003) Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia **17**, 17–25
195 Kofler, R., Schmidt, S., Koller, A. and Ausserlechner, M. J. (2003) Resistance to glucocorticoid-induced apoptosis in lymphoblastic leukemia. J. Endocrinol. **178**, 19–27
196 Gaynon, P. S. and Carrel, A. L. (1999) Glucocorticosteroid therapy in childhood acute lymphoblastic leukemia. Adv. Exp. Med. Biol. **457**, 593–605
197 Moalli, P. A. and Rosen, S. T. (1994) Glucocorticoid receptors and resistance to glucocorticoids in hematologic malignancies. Leuk. Lymphoma **15**, 363–374
198 Myers, D. E., Chardan-Langlie, M., Chelstrom, L. M. and Uckun, F. M. (1996) *In vitro* and *in vivo* anti-leukemic efficacy of cyclic AMP modulating agents against human leukemic B-cell precursors. Leuk. Lymphoma **22**, 259–264

199 Ogawa, R., Streiff, M. B., Bulgayemic, A. and Katb, G. J. (2002) Inhibition of PDE4 phosphodiesterase activity induces growth suppression, apoptosis, glucocorticoid sensitivity, p53, and p21$^{WAF1/CIP1}$ proteins in human acute lymphoblastic leukemia cells. Blood **99**, 3390–3397

200 Shen, Z. X., Shi, Z. Z., Fang, J., Gu, B. W., Li, J. M., Zhu, Y. M., Shi, J. Y., Zheng, P. Z., Yan, H., Liu Y. F. et al. (2004) All-*trans* retinoic acid/As_2O_3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. Proc. Natl. Acad. Sci. U.S.A. **101**, 5328–5335

201 Hayakawa, F. and Privalsky, M. L. (2004) Phosphorylation of PML by mitogen-activatec protein kinases plays a key role in arsenic trioxide-mediated apoptosis. Cancer Cell **5**, 389–401

202 Olsson, I. L. and Breitman, T. R. (1982) Induction of differentiation of the human histiocytic lymphoma cell line U-937 by retinoic acid and cyclic adenosine 3′:5′-monophosphate-inducing agents. Cancer Res. **42**, 3924–3927

203 Yang, L., Zhao, H., Li, S. W., Ahrens, K., Collins, C., Eckenrode, S., Ruan, Q. G., McIndoe, R. A. and She, J. X. (2003) Gene expression profiling during all-*trans* retinoic acid-induced cell differentiation of acute promyelocytic leukem a cells. J. Mol. Diagn. **5**, 212–221

204 Zhu, Q., Zhang, J. W., Zhu, H. Q., Shen, Y. L., Flexor, M., Jia, P. M., Yu, Y., Cai, X., Waxman, S., Lanotte, M. et al. (2002) Synergic effects of arsenic trioxide and cAMP during acute promyelocytic leukemia cell maturation sublends a novel signaling cross-talk. Blood **99**, 1014–1022

205 Stegmaier, K., Ross, K. N., Colavito, S. A., O'Malley, S., Stockwell, B. R. and Golub, T. R. (2004) Gene expression-based high-throughput screening (GE-HTS) and application to leukemia differentiation. Nat. Genet. **36**, 257–263

206 Guillemin, M. C., Raffoux, E., Vitoux, D., Kogan, S., Scillhi, H., Lallemand-Breitenbach, V., Zhu, J., Janin, A., Daniel, M. T., Gourmel, B. et al. (2002) *In vivo* activation of cAMP signaling induces growth arrest and differentiation in acute promyelocytic leukemia. J. Exp. Med. **196**, 1373–1380

207 Lamb, D. and Steinberg, R. A. (2002) Anti-proliferative effects of 8-chloro-cAMP and other cAMP analogs are unrelated to their effects on protein kinase A regulatory subunit expression. J. Cell. Physiol. **192**, 216–224

208 Parrella, E., Gianni, M., Cecconi, V., Nigro E., Barzago, M. M., Rambaldi, A., Rochette-Egly, C., Terao, M. and Garattini, E. (2004) Phosphodiesterase IV inhibition by piclamilas: potentiates the cytodifferentiating action of retino ds in myeloid leukemia cells: cross-talk between the cAMP and the ret noic acid signaling pathways. J. Biol. Chem. **279**, 42026–42040

209 Garcia-Bermejo, L., Perez, C., Vilaboa, N. E., de Blas, E. and Aller, P. (1998) cAMP increasing agents attenuate the generation of apoptosis by etoposide in promonocytic leukemia cells. J. Cell Sci. **111**, 637–644

210 Zhu, W. H., Majluf-Cruz, A. and Ornburo, G. A. (1998) Cyclic AMP-specific phosphodiesterase inhibitor rolipram and RO-20-1724 promoted apoptosis in HL60 promyelocytic leukemic cells via cyclic AMP-independent mechanism. Life Sci. **63**, 265–274

211 Pae, H. O., Yoo, J. C., Jun, C. D., Paik, S. G., Choi, B. M., Baek, K. H., Kim, J. M. and Chung, H. T. (1999) Increased intracellular cAMP renders HL-60 cells resistant to cytotoxicity of taxol. Immunopharmacol. Immunotoxicol. **21**, 233–245

212 Tortorella, C., Piazzolla, G., Spaccavento, F. and Antonaci, S. (1998) Effects of granulocyte–macrophage colony-stimulating factor and cyclic AMP interaction on human neutrophil apoptosis. Mediators Inflamm. **7**, 391–396

213 Martin, M. C., Dransfield, I., Haslett, C. and Rossi, A. G. (2001) Cyclic AMP regulation of neutrophil apoptosis occurs via a novel protein kinase A-independent signaling pathway. J. Biol. Chem. **276**, 45041–45050

214 Jimenez, J. L., Iniguez, M. A., Munoz-Fernandez, M. A. and Fresno, M. (2004) Effect of phosphodiesterase 4 inhibitors on NFAT-dependent cyclooxygenase-2 expression in human T lymphocytes. Cell Signalling **16**, 1363–1373

215 Ito, C., Ribeiro, R. C., Behm, F. G., Raimondi, S. C., Pui, C. H. and Campana, D. (1998) Cyclosporin A induces apoptosis in childhood acute lymphoblastic leukemia cells. Blood **91**, 1001–1007

216 Hashimoto, Y., Matsuoka, N., Kawakami, A., Tsuboi, M., Nakashima, T., Eguchi, K., Tomioka, T. and Kanematsu, T. (2001) Novel immunosuppressive effect of FK506 by augmentation of T cell apoptosis. Clin. Exp. Immunol. **125**, 19–24

217 Zwain, I. H. and Amato, P. (2001) cAMP-induced apoptosis in granulosa cells is associated with up-regulation of P53 and bax and down-regulation of clusterin. Encocr. Res. **27**, 233–249

218 Huston, E., Beard, M., McCallum, F., Pyne, N. J., Vandenabeele, P., Scotland, G. and Houslay, M. D. (2000) The cAMP-specific phosphodiesterase PDE4A5 is cleaved downstream of its SH3 interaction domain by caspase-3: consequences for altered intracellular distribution. J. Biol. Chem. **275**, 28063–28074

219 Ding, B., Abe, J., Wei, H., Huang, Q., Walsh, R. A., Molina, C. A., Zhao, A., Sadoshima, J., Blaxall, B. C., Berk, B. C. and Yan, C. (2005) Functional role of phosphodiesterase 3 in cardiomyocyte apoptosis: implication in heart failure. Circulation **111**, 2465–2476

220 Kwon, G., Pappan, K. L., Marshall, C. A., Schaller, J. E. and McDaniel, M. L. (2004) cAMP dose-dependently prevents palmitate-induced apoptosis by both protein kinase A- and cAMP-guanine nucleot de exchange factor-dependent pathways in β-cells. J. Biol. Chem. **279**, 8938–8945

221 Cullen, K. A., McCool, J., Anwer, M. S. and Webster, C. R. (2004) Activation of cAMP-guanine exchange factor confers PKA-independent protection from hepatocyte apoptosis. Am. J. Physiol. Gastrointest. Liver Physiol. **287**, G334–G343

222 Nishihara, H., Kizaka-Kondoh, S., Insel, P. A. and Eckmann, L. (2003) Inhibition of apoptosis in normal and transformed intestinal epithelial cells by cAMP through induction of inhibitor of apoptosis protein (IAP)-2. Proc. Natl. Acad. Sci. U.S.A. **100**, 8921–8926

223 Hoshiro, T., Tsutsumi, S., Tomsato, W., Hwang, H. J., Tsuchiya, T. and Mizushima, T. (2003) Prostaglandin E_2 protects gastric mucosal cells from apoptosis via EP_2 and EP_4 receptor activation. J. Biol. Chem. **278**, 12752–12758

224 Chin, P. C. and D'Mello, S. R. (2005) Brain chemotherapy from the bench to the clinic: targeting neuronal survival with small molecule inhibitors of apoptosis. Front. Biosci. **10**, 552–568

225 Zhou, B., Li, F., Chen, H. and Song, J. (2005) The modulation of apoptosis by cyclic AMP involves Akt and epidermal growth factor receptor. Int. J. Biochem. Cell Biol. **37**, 1483–1495

226 Machida, K., Inoue, H., Matsumoto, K., Tsuda, M., Fukuyama, S., Koto, H., Aizawa, H., Kureishi, Y., Hara, N. and Nakanishi, Y. (2005) Activation of PI3K-Akt pathway mediates antiapoptotic effects of β-adrenergic agonist in airway eosinophils. Am. J. Physiol. Lung Cell. Mol. Physiol. **288**, L860–L867

227 Wang, W., Masu, K., Tamura, G., Suzuki, K., Ohwada, K., Okuyama, K., Shirato, K., Takayanagi, M. and Ohno, I. (2005) Inhibition of eosinophil survival by a selective inhibitor of phosphodiesterase 4 via the induction of apoptosis. Biol. Pharm Bull. **28**, 515–519

228 Frame, M., Wan, K. F., Tate, R., Vandenabeele, P. and Pyne, N. J. (2001) The γ subunit of the rod photoreceptor cGMP phosphodiesterase can modulate the proteolysis of two cGMP binding cGMP-specific phosphodiesterases (PDE6 and PDE5) by caspase-3. Cell. Signalling **13**, 735–741

229 Frame, M. J., Tate, R., Adams, D. R., Morgan, K. M., Houslay, M. D., Vandenabeele, P. and Pyne, N. J. (2003) Interaction of caspase-3 with the cyclic GMP binding cyclic GMP specific phosphodiesterase (PDE5a1). Eur. J. Biochem. **270**, 962–970

230 Gledhill, R. M. (1978) Differential sensitivities of normal human and chronic lymphocytic leukemia lymphocytes to 1-methyl,3-isobutylxanthine and N^6-2′-o-dibutyryl adenosine-3′,5′-cyclic monophosphate. J. Med. **9**, 291–311

231 Huang, R. W., Tsuda, H. and Takatsuki, K. (1993) Interleukin-2 prevents programmed cell death in chronic lymphocytic leukemia cells. Int. J. Hematol. **58**, 83–92

232 Mentz, F., Merle-Beral, H., Ouaaz, F. and Binet, J. L. (1995) Theophylline, a new inducer of apoptosis in B-CLL: role of cyclic nucleotides. Br. J. Haematol. **90**, 957–959

233 Mentz, F., Mossalayi, M. D., Ouaaz, F., Baudet, S., Issaly, F., Klorza, S., Semichon, M., Binet, J. L. and Merle-Beral, H. (1996) Theophylline synergizes with chlorambucil in inducing apoptosis of B-chronic lymphocytic leukemic cells. Blood **88**, 2172–2182

234 Mentz, F., Merle-Beral, H. and Dalloul, A. H. (1999) Theophylline-induced B-CLL apoptosis is partly dependent on cyclic AMP production but independent of CD38 expression and endogenous IL-10 production. Leukemia **13**, 78–84

235 Wiernik, P. H., Paietta, E., Goloubeva, O., Lee, S. J., Makower, D., Bennett, J. M., Wade, J. L., Ghosh, C., Kaminer, L. S., Pizzolo, J. and Tallman, M. S. (2004) Phase II study of theophylline in chronic lymphocytic leukemia: a study of the Eastern Cooperative Oncology Group (E4998). Leukemia **18**, 1625–1630

236 Kim, C. H. and Lerner, A. (1998) Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. Blood **92**, 2484–2494

237 Sarfati, M., Mateo, V., Baudet, S., Rubio, M., Fernandez, C., Dav, F., Binet, J. L., Delic, J. and Merle-Beral, H. (2003) Sildenafil and vardenafil, types 5 and 6 phosphodiesterase inhibitors, induce caspase-dependent apoptosis of B-chronic lymphocytic leukemia cells. Blood **101**, 265–269

238 Saenz de Tejada, I., Angulo, J., Cuevas, P., Fernandez, A., Moncada, I., Allona, A., Lledo, E., Korschen, H. G., Niewohner, U., Haning, H. et al. (2001) The phosphodiesterase inhibitory selectivity and the *in vitro* and *in vivo* potency of the new PDE5 inhibitor vardenafil. Int. J. Impotence Res. **13**, 282–290

239 Ballard, S. A., Gingell, C. J., Tang, K., Turner, L. A., Price, M. E. and Naylor, A. M. (1998) Effects of sildenafil on the relaxation of human corpus cavernosum tissue *in vitro* and on the activities of cyclic nucleotide phosphodiesterase isozymes. J. Urol. **159**, 2164–2171

240 Loughney, K., Hill, T. R., Florio, V. A., Uher, L., Rosman, G. J., Wolda, S. L., Jones, B. A., Howard, M. L., McAllister-Lucas, L. M., Sonnenburg, W. K. et al. (1998) Isolation and characterization of cDNAs encoding PDE5A, a human cGMP-binding, cGMP-specific 3′,5′-cyclic nucleotide phosphodiesterase. Gene **216**, 139–147

241 Wang, P., Wu, P., Myers, J. G., Stamford, A., Egan, R. W. and Billah, M. M. (2001) Characterization of human, dog and rabbit corpus cavernosum type 5 phosphodiesterases. Life Sci. **68**, 1977–1987

242 Muradov, K. G., Granovsky, A. E. and Artemyev, N. O. (2003) Mutation in rod PDE6 linked to congenital stationary night blindness impairs the enzyme inhibition by its γ-subunit. Biochemistry **42**, 3305–3310

243 Chen, J. C., Chen, J. Q., Xie, Q. M. and Zhu, Y. L. (2004) Selective inhibition of purified human phosphodiesterase 4A expressed in yeast cell GL62 by ciclamilast, piclamilast, and roflumilast. Acta Pharmacol. Sin. **25**, 1171–1175

244 Shipp, M. A., Ross, K. N., Tamayo, P., Weng, A. P., Kutok, J. L., Aguiar, R. C., Gaasenbeek, M., Angelo, M., Reich, M., Pinkus, G. S. et al. (2002) Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat. Med. **8**, 68–74

245 Alizadeh, A. A., Eisen, M. B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., Boldrick, J. C., Sabet, H., Tran, T., Yu, X. et al. (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature (London) **403**, 503–511

246 Fernandez, P. M., Brunet, F., Jimenez, M. A., Saez, J. M., Cereghini, S. and Zakin, M. M. (2000) Nuclear receptors Nor1 and NGFI-B/Nur77 play similar, albeit distinct, roles in the hypothalamo-pituitary-adrenal axis. Endocrinology **141**, 2392–2400

247 Dave, S. S., Wright, G., Tan, B., Rosenwald, A., Gascoyne, R. D., Chan, W. C., Fisher, R. I., Braziel, R. M., Rimsza, L. M., Grogan, T. M. et al. (2004) Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. N. Engl. J. Med. **351**, 2159–2169

248 Siegmund, B., Welsch, J., Loher, F., Meinhardt, G., Emmerich, B., Endres, S. and Eigler, A. (2001) Phosphodiesterase type 4 inhibitor suppresses expression of anti-apoptotic members of the Bcl-2 family in B-CLL cells and induces caspase-dependent apoptosis. Leukemia **15**, 1564–1571

249 Moon, E. Y. and Lerner, A. (2003) PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. Blood **101**, 4122–4130

250 Tiwari, S., Felekkis, K., Moon, E. Y., Flies, A., Sherr, D. H. and Lerner, A. (2004) Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1, but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. Blood **103**, 2661–2667

251 de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A. and Bos, J. L. (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature (London) **396**, 474–477

252 Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E. and Graybiel, A. M. (1998) A family of cAMP-binding proteins that directly activate Rap1. Science **282**, 2275–2279

253 Enserink, J. M., Christensen, A. E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H. G., Doskeland, S. O., Blank, J. L. and Bos, J. L. (2002) A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat. Cell Biol. **4**, 901–906

254 Enserink, J. M., Price, L. S., Methi, T., Mahic, M., Sonnenberg, A., Bos, J. L. and Tasken, K. (2004) The cAMP-Epac-Rap1 pathway regulates cell spreading and cell adhesion to laminin-5 through the $\alpha 3\beta 1$ integrin but not the $\alpha 6\beta 4$ integrin. J. Biol. Chem. **279**, 44883–44895

255 Kang, G., Joseph, J. W., Chepurny, O. G., Monaco, M., Wheeler, M. B., Bos, J. L., Schwede, F., Genieser, H. G. and Holz, G. G. (2003) Epac-selective cAMP analog 8-pCPT-2′-O-Me-cAMP as a stimulus for Ca^{2+}-induced Ca^{2+} release and exocytosis in pancreatic β-cells. J. Biol. Chem. **278**, 8279–8285

256 Klein, U., Tu, Y., Stolovitzky, G. A., Mattioli, M., Cattoretti, G., Husson, H., Freedman, A., Inghirami, G., Cro, L., Baldini, L., Neri, A., Califano, A. and Dalla-Favera, R. (2001) Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J. Exp. Med. **194**, 1625–1638

257 Kiefer, J., Okret, S., Jondal, M. and McConkey, D. J. (1995) Functional glucocorticoid receptor expression is required for cAMP-mediated apoptosis in a human leukemic T cell line. J. Immunol. **155**, 4525–4526

258 Doucas, V., Shi, Y., Miyamoto, S., West, A., Verma, I. and Evans, R. M. (2000) Cytoplasmic catalytic subunit of protein kinase A mediates cross-repression by NF-κB and the glucocorticoid receptor. Proc. Natl. Acad. Sci. U.S.A. **97**, 11893–11898

259 Gruol, D. J., Campbell, N. F. and Bourgeois, S. (1986) Cyclic AMP-dependent protein kinase promotes glucocorticoid receptor function. J. Biol. Chem. **261**, 4909–4914

260 Gruol, D. J. and Altschmied, J. (1993) Synergistic induction of apoptosis with glucocorticoids and 3′,5′-cyclic adenosine monophosphate reveals agonist activity by RU 486. Mol. Endocrinol. **7**, 104–113

261 McConkey, D. J., Orrenius, S., Okret, S. and Jondal, M. (1989) Cyclic AMP potentiates glucocorticoid-induced endogenous endonuclease activation in thymocytes. FASEB J. **7**, 580–585

262 Miller, A. H., Vogt, G. J. and Pearce, B. D. (2002) The phosphodiesterase type 4 inhibitor, rolipram, enhances glucocorticoid receptor function. Neuropsychopharmacology **27**, 939–948

263 Refojo, D., Liberman, A. C., Giacomini, D., Carbia Nagashima, A., Graciarena, M., Echenique, C., Paez Pereda, M., Stalla, G., Holsboer, F. and Arzt, E. (2003) Integrating systemic information at the molecular level: cross-talk between steroid receptors and cytokine signaling on different target cells. Ann. N.Y. Acad. Sci. **992**, 196–204

264 Reichardt, H. M., Tuckermann, J. P., Gottlicher, M., Vujic, M., Weih, F., Angel, P., Herrlich, P. and Schutz, G. (2001) Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. EMBO J. **20**, 7168–7173

265 Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P. and Schutz, G. (1998) DNA binding of the glucocorticoid receptor is not essential for survival. Cell **93**, 531–541

266 Helmberg, A., Auphan, N., Caelles, C. and Karin, M. (1995) Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. EMBO J. **14**, 452–460

267 Manganiello, V. and Vaughan, M. (1972) An effect of dexamethasone on adenosine 3′,5′-monophosphate content and adenosine 3′,5′-monophosphate phosphodiesterase activity of cultured hepatoma cells. J. Clin. Invest. **51**, 2763–2767

268 Hermsdorf, T., Richter, W. and Dettner, D. (1999) Effects of dexamethasone and glucagon after long-term exposure on cyclic AMP phosphodiesterase 4 in cultured rat hepatocytes. Cell. Signalling **11**, 685–690

269 Ahlstrom, M., Pekkinen, M., Huttunen, M. and Lamberg-Allardt, C. (2005) Dexamethasone down-regulates cAMP-phosphodiesterase in human osteosarcoma cells. Biochem. Pharmacol. **69**, 267–275

270 Crocker, I. C., Church, M. K., Ohla, S. E. and Townley, R. G. (2000) Beclomethasone decreases elevations in phosphodiesterase activity in human T lymphocytes. Int. Arch. Allergy Immunol. **121**, 151–160

271 Baus, E., Van Laethem, F., Andris, F., Rolin, S., Urbain, J. and Lao, O. (2001) Dexamethasone increases intracellular cyclic AMP concentration in murine T lymphocyte cell lines. Steroids **66**, 39–47

272 Wang, J. H., Sharma, R. K. and Moolbroek, M. J. (1990) Calmodulin-stimulated cyclic nucleotide phosphodiesterases. In Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action (Beavo, J. and Housley, M. D., eds.), pp. 19–59, John Wiley & Sons, Chichester

273 Sharma, R. K. and Rickie, R. A. (1996) Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1). In Phosphodiesterase Inhibitors (Schudt, C., Dent, G. and Rabe, K. F., eds.), pp. 65–80, Academic Press, London

274 Yan, C., Zhao, A. Z., Bentley, J. K., Loughney, K., Ferguson, K. and Beavo, J. A. (1995) Molecular cloning and characterization of a calmodulin-dependent phosphodiesterase enriched in olfactory sensory neurons. Proc. Natl. Acad. Sci. U.S.A. **92**, 9677–9681

275 Loughney, K., Martins, T. J., Harris, E. A., Sadhu, K., Hicks, J. B., Sonnenburg, W. K., Beavo, J. A. and Ferguson, K. (1995) Isolation and characterization of cDNAs corresponding to two human calcium, calmodulin-regulated, 3′,5′-cyclic nucleotide phosphodiesterases. J. Biol. Chem. **271**, 796–806

276 Yu, J., Welda, S. L., Frazier, A. L., Florio, V. A., Martins, T. J., Snyder, P. B., Harris, E. A., McCaw, K. N., Farrell, C. A., Steiner, B. et al. (1997) Identification and characterisation of a human calmodulin-stimulated phosphodiesterase PDE1B1. Cell. Signalling **9**, 519–529

277 Manganiello, V. C., Tanaka, T. and Murashima, S. (1990) Cyclic GMP-stimulated cyclic nucleotide phosphodiesterases. In Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action (Beavo, J. and Housley, M. D., eds.), pp. 61–85, John Wiley & Sons, Chichester

278 Manganiello, V. C., Smith, C. J., Degerman, E. and Belfrage, P. (1990) Cyclic GMP-inhibited cyclic nucleotide phosphodiesterases. In Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action (Beavo, J. and Housley, M. D., eds.), pp. 87–115, John Wiley & Sons, Chichester

279 Komas, N., Movsesian, M., Kedov, S., Degerman, E., Belfrage, P. and Manganiello, V. (1996) cGMP-inhibited phosphodiesterases (PDE3). In Phosphodiesterase Inhibitors (Schudt, C., Dent, G. and Rabe, K. F., eds.), pp. 89–110, Academic Press, London

280 Epstein, P. M., Strada, S. J., Sarada, K. and Thompson, W. J. (1982) Catalytic and kinetic properties of purified high-affinity cyclic AMP phosphodiesterase from dog kidney. Arch. Biochem. Biophys. **218**, 119–133

281 Conti, M. and Swinnen, J. V. (1990) Structure and function of the Rolipram-sensitive low-K_m cyclic AMP phosphodiesterases: a family of highly related enzymes. In Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action (Beavo, J. and Houslay, M. D., eds.), pp. 243–265, John Wiley & Sons, Chichester

282 Conti, M., Nemoz, G., Sette, C. and Vicini, E. (1995) Recent progress in understanding the hormonal regulation of phosphodiesterases. Endocr. Rev. **16**, 370–389

283 Wallace, D. A., Johnston, L. A., Huston, E., MacMaster, D., Houslay, T. M., Cheung, Y. F., Campbell, L., Miller, J. E., Smith, R. A., Gall, I. et al. (2005) Identification and characterization of PDE4A11, a novel, widely expressed long isoform encoded by the human PDE4A cAMP phosphodiesterase gene. Mol. Pharmacol. **67**, 1920–1934

284 Francis, S. H., Thomas, M. K. and Corbin, J. D. (1990) Cyclic GMP-binding cyclic GMP-specific phosphodiesterase from lung. In Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action (Beavo, J. and Houslay, M. D., eds.), pp. 117–139, John Wiley & Sons, Chichester
285 Kotera, J., Fujishige, K., Akatsuka, H., Imai, Y., Yanaka, N. and Omori, K. (1998) Novel alternative splice variants of cGMP-binding cGMP-specific phosphodiesterase. J. Biol. Chem. **273**, 26982–26990
286 Lin, C. S., Lau, A., Tu, R. and Lue, T. F. (2000) Expression of three isoforms of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in human penile cavernosum. Biochem. Biophys. Res. Commun. **268**, 628–635
287 Bischoff, E. (2004) Potency, selectivity, and consequences of nonselectivity of PDE inhibition. Int. J. Impotence Res. **16** (suppl. 1), S11–S14
288 Gillespie, P. G. (1990) Phosphodiesterases in visual transduction by rods and cones. In Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action (Beavo, J. and Houslay, M. D., eds.), pp. 163–183, John Wiley & Sons, Chichester
289 Gillespie, P. G. and Beavo, J. A. (1989) Inhibition and stimulation of photoreceptor phosphodiesterases by dipyridamole and M&B 22,948. Mol. Pharmacol. **36**, 773–781
290 Han, P., Zhu, X. and Michaeli, T. (1997) Alternative splicing of the high affinity cAMP-specific phosphodiesterase (PDE7A) mRNA in human skeletal muscle and heart. J. Biol. Chem. **272**, 16152–16157
291 Wang, P., Wu, P., Egan, R. W. and Billah, M. M. (2000) Cloning, characterization, and tissue distribution of mouse phosphodiesterase 7A1. Biochem. Biophys. Res. Commun. **276**, 1271–1277
292 Sasaki, T., Kotera, J., Yuasa, K. and Omori, K. (2000) Identification of human PDE7B, a cAMP-specific phosphodiesterase. Biochem. Biophys. Res. Commun. **271**, 575–583
293 Wang, P., Wu, P., Egan, R. W. and Billah, M. M. (2003) Identification and characterization of a new human type 9 cGMP-specific phosphodiesterase splice variant (PDE9A5): differential tissue distribution and subcellular localization of PDE9A variants. Gene **314**, 15–27
294 Fujishige, K., Kotera, J. and Omori, K. (1999) Striatum- and testis-specific phosphodiesterase PDE10A isolation and characterization of a rat PDE10A. Eur. J. Biochem. **266**, 1118–1127
295 Robison, G. A., Butcher, R. W. and Sutherland, E. W. (1971) Cyclic AMP, Academic Press, New York
296 Taskén, K. and Aandahl, E. M. (2004) Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol. Rev. **84**, 137–167
297 Kaupp, U. B. and Seifert, R. (2002) Cyclic nucleotide-gated ion channels. Physiol. Rev. **82**, 769–824
298 Staples, K. J., Bergmann, M., Tomita, K., Houslay, M. D., McPhee, I., Barnes, P. J., Giembycz, M. A. and Newton, R. (2001) Adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent inhibition of IL-5 from human T lymphocytes is not mediated by the cAMP-dependent protein kinase A. J. Immunol. **167**, 2074–2080
299 Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P. and Ghosh, S. (1997) The transcriptional activity of NF-κB is regulated by the IκB-associated PKAc subunit through a cyclic AMP-independent mechanism. Cell **89**, 413–424
300 Kraus-Friedmann, N. (2000) Cyclic nucleotide-gated channels in non-sensory organs. Cell Calcium **27**, 127–138
301 De Cesare, D. and Sassone-Corsi, P. (2000) Transcriptional regulation by cyclic AMP-responsive factors. Prog. Nucleic Acid Res. Mol. Biol. **64**, 343–369
302 Bodor, J., Bodorova, J. and Gress, R. E. (2000) Suppression of T cell function: a potential role for transcriptional repressor ICER. J. Leukocyte Biol. **67**, 774–779
303 Cho, E. S., Yu, J. H., Kim, M. S. and Yim, M. (2005) Rolipram, a phosphodiesterase 4 inhibitor, stimulates inducible cAMP early repressor expression in osteoblasts. Yonsei Med. J. **46**, 149–154
304 Semizarov, D., Glesne, D., Laouar, A., Schlebel, K. and Huberman, E. (1998) A lineage-specific protein kinase crucial for myeloid maturation. Proc. Natl. Acad. Sci. U.S.A. **95**, 15412–15417
305 Zimmermann, B., Chiorini, J. A., Ma, Y., Kotin, R. M. and Herberg, F. W. (1999) PrKX is a novel catalytic subunit of the cAMP-dependent protein kinase regulated by the regulatory subunit type I. J. Biol. Chem. **274**, 5370–5378
306 Rena, G., Prescott, A. R., Guo, S., Cohen, P. and Unterman, T. G. (2001) Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-13-3 binding, transactivation and nuclear targetting. Biochem. J. **354**, 605–612
307 Frame, S. and Cohen, P. (2001) GSK3 takes centre stage more than 20 years after its discovery. Biochem. J. **359**, 1–16

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EXHIBIT B

PDE4 inhibitors augment levels of glucocorticoid receptor in B cell chronic lymphocytic leukemia but not in normal circulating hematopoietic cells

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Abstract

Type 4 cAMP phosphodiesterase (PDE4) inhibitors, a class of compounds in clinical development that activate cAMP-mediated signaling by inhibiting cAMP catabolism, offer a feasible means by which to potentiate glucocorticoid-mediated apoptosis in lymphoid malignancies such as B cell chronic lymphocytic leukemia (B-CLL). In this study, we demonstrate that PDE4 inhibitors up-regulate glucocorticoid receptor (GRα) transcript levels in B-CLL cells but not T-CLL cells or Sezary cells or normal circulating T cells, B cells, monocytes or neutrophils. As GRα transcript half-life does not vary in CLL cells treated with rolipram, the four-fold increase in GRα mRNA levels observed within four hours of rolipram treatment appears to result from an increase in GRα transcription. Rolipram treatment increases levels of transcripts derived from the 1A3 promoter to a greater extent than the 1B promoter. Treatment of B-CLL cells with two PDE4 inhibitors currently in clinical trials also augments glucocorticoid receptor transcript levels and glucocorticoid-mediated apoptosis. Washout studies demonstrate that treatment with both drug classes irreversibly augments apoptosis over the same time frame that glucocorticoid receptor up-regulation occurs. While treatment of B-CLL cells with glucocorticoids reduces basal GRα transcript levels in a dose-related manner, co-treatment with rolipram results in GRα transcript levels above baseline that are intermediate between those observed following treatment with either drug alone. Our results suggest that as a result of their unusual sensitivity to PDE4 inhibitor-mediated up-regulation of GRα expression, treatment of B-CLL patients with combined PDE4 inhibitor/glucocorticoid therapy may be of therapeutic benefit in this disease.

Introduction

Glucocorticoids are an important component of standard therapy for several lymphoid malignancies, including multiple myeloma, acute lymphoblastic leukemia and diffuse large B cell lymphoma. As early studies in patients with B cell chronic lymphocytic leukemia (B-CLL) demonstrated that addition of prednisone to chlorambucil augmented response rate but not median survival, glucocorticoids are not generally a standard component of initial therapy for patients with B-CLL [1,2]. Nonetheless, two studies of high dose glucocorticoid therapy have suggested that glucocorticoids can be of clinical benefit to a subset of patients with treatment-refractory B-CLL [3,4]. Despite frequent responses to glucocorticoid treatment, monotherapy with glucocorticoids is not curative in any lymphoid malignancy, but the mechanisms underlying clinical glucocorticoid resistance remain controversial. Structural alterations in the GR are commonly identified in lymphoid cell lines that have been selected for glucocorticoid resistance by prolonged culture in dexamethasone, but comparable alterations in primary malignant lymphoid cells have been only infrequently reported [5-9]. A detailed analysis of treated B-CLL patients failed to identify abnormalities in either the DNA or steroid binding domains of leukemic GRs [10]. Non-structural modifications of glucocorticoid signaling pathways are likely to be important in clinical glucocorticoid resistance and efforts to identify and reverse these modifications may be therapeutically useful [8,9].

cAMP-mediated signaling can favorably alter the apoptotic response to glucocorticoids in specific lymphoid subsets, although the precise molecular explanation for this relationship remains unclear. Seminal early work done by Suzanne Bourgeois and colleagues demonstrated that isolation of Wehi-7 cells, a murine T cell lymphoma line, that were resistant to cAMP-mediated apoptosis due to alterations in protein kinase A made subsequent development of spontaneous glucocorticoid-resistant cells occur at higher frequencies (10^{-7}) than in wildtype cells (> 10^{-10}) [11]. Gruol and Altschmied subsequently determined that RU486, ordinarily a GR antagonist for GC-induced lymphoid cytolysis, becomes an agonist in the setting of co-treatment with a cAMP analogue [12]. Conversely, McConkey and colleagues reported that a glucocorticoid receptor (GR) deficient ICR.27 cells, a variant of the CEM T cell lymphoma line, are insensitive to cAMP-induced apoptosis. Transfection of ICR.27 cells with the glucocorticoid receptor restored sensitivity to cAMP-mediated apoptosis [13]. Finally, the catalytic subunit of PKA has been shown to associate with the glucocorticoid receptor [14].

One critical factor that regulates lymphoid sensitivity to glucocorticoids is the level of GR expression. Gruol et al determined that treatment of Wehi-7 cells with cAMP analogues raised glucocorticoid receptor transcript and protein levels [15]. Several mechanisms have been proposed to explain why GR transcript levels rise following treatment of specific cell subsets with agents that augment cAMP signaling. In studies of rat hepatoma cells,

Dong et al reported that treatment with 8-bromo-cAMP increases GR mRNA half-life from 4 hours to 10 hours [16]. As treatment of such cell cultures with inhibitors of protein or mRNA synthesis had no effect on the ability of 8-bromo-cAMP to increase GR transcript levels, Dong et al argue that a principal mechanism by which cAMP signaling augments GR transcript levels must be through GR mRNA stabilization. In contrast, utilizing transfection of GR promoter luciferase constructs into HeLa cells, Penuelas et al determined that treatment with the adenylate cyclase activator forskolin doubled the transcriptional activity of the human GR promoter [17]. After mapping and testing five putative CRE binding sites, the authors demonstrated loss of forskolin inducibility in promoter constructs shorter than 1 kB and the presence of a CRE element which bound CRE in vitro in shift assays. Thus, it appears that in some cell lineages, cAMP-induced augmentation of GR transcript levels is due to augmented transcription rather than mRNA stabilization.

Type 4 cAMP phosphodiesterase (PDE4) inhibitors offer a therapeutically plausible means by which to take advantage of the phenomenon of cAMP-mediated augmentation of glucocorticoid sensitivity in malignant lymphoid cells. The PDE4 family play a key role in catabolizing cAMP in a variety of human hematopoietic cells and PDE4 inhibitors are in late stage clinical studies for a variety of inflammatory illnesses, including asthma and chronic obstructive disease [18]. In prior work, we have determined that inhibition of PDE4, in the absence of addition of exogenous adenylate cyclase activators such as forskolin or beta adrenergic agonists, increases cAMP levels, activates protein kinase A as judged by CREB phosphorylation, and induces apoptosis in primary B-CLL cells, albeit in well less than 100% of cells [19]. Treatment with the prototypic PDE4 inhibitor rolipram induces mitochondrial release of cytochrome c, activation of caspase 9 and 3, and cleavage of PARP in CLL cells [20]. PDE4 inhibitors also activate Rap1 in B-CLL cells due to cAMP-mediated activation of the Rap1 GDP exchange factor EPAC1, but EPAC activation appears to be anti-apoptotic [21]. PDE4 inhibitors thus induce both PKA-mediated pro-apoptotic and EPAC-mediated anti-apoptotic signaling pathways in B-CLL cells, with the PKA-mediated pro-apoptotic pathway having a dominant effect.

PDE4 inhibitors such as rolipram augment hydrocortisone or dexamethasone-induced apoptosis in primary B-CLL cells, as well as transactivation of glucocorticoid response element (GRE)-containing reporter constructs [22]. Both of these effects are reversed by the type I PKA antagonist *Rp*-8Br-cAMPS. The specific mechanism or mechanisms by which PDE4 inhibitors increase glucocorticoid sensitivity in B-CLL cells remain unknown. In this study, we sought to determine whether PDE4 inhibitors alter leukemic expression of glucocorticoid receptor. We find that PDE4 inhibitors augment expression of GRα at a transcriptional level and that among human primary hematopoietic cells, this effect is quite specific to B-CLL.

Methods and Materials

Materials: The following reagents were obtained from commercial sources: R-(-)-Rolipram (Biomol, Plymouth Meeting PA), forskolin (Sigma, St. Louis MO), 1-Methyl-3-isobutylxanthine [IBMX] (Sigma), actinomycin D (Calbiochem, San Diego CA). Cilomilast and roflumilast were obtained from Memory Pharmaceuticals (Montvale, NJ).

Cell Culture and Isolation: Blood samples were obtained in heparinized tubes with IRB-approved consent from flow cytometry-confirmed B-CLL patients that were either untreated or for whom at least 1 month had elapsed since chemotherapy. Patients with active infections or other serious medical conditions were not included in this study. Patients with white blood cell counts of less than 15,000/μl by automated analysis were excluded from this study. Whole blood was layered on Ficoll-Hystopaque (Sigma) and peripheral blood mononuclear cells (PBMC) isolated after centrification. PBMC were washed and resuspended at $1x10^7$ cells/ml in complete media [RPMI-1640 (Mediatech) supplemented with 10% fetal bovine serum (Sigma), 20mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Mediatech)]. PBMC was found to contain >90% B-CLL by FACS without additional purification. Normal B cells, T cells, and monocytes were obtained from anonymous healthy donors (New York Biologics, Southampton NY) and isolated via magnetic negative depletion per the manufacturer's protocol (Miltenyi, Bergisch-Gladbach Germany) from PBMC. Polymorphonuclear cells (PMNs) were obtained by erythrocyte depletion of whole blood via dextran sedimentation followed by removal of PBMC using Ficoll seperation. With the exception of PMNs, which were used immediately after purification, all other primary normal and malignant cell populations were rested overnight at 37°C prior to use.

Western analysis: Following cell culture, cells were collected by centrifugation (400xg; 10 min.), washed once with phosphate buffered saline and lysed in ice-cold 10 mM HEPES-NaOH buffer (pH 7.4) containing 1%TritonX-100, 10% glycerol, 25 mM β-glycerophosphate, 100 mM NaCl, 2mM EDTA, 2mM EGTA, 1mM dithiothriotol, 1 mM vanadate, 1mM phenylmethanesulfonyl fluoride, and 1mM benzamidine. Cell lysates were transferred to 1.5 mL tubes and centrifuged at 14,000 rpm for 30 minutes (5°C) in a microcentrifuge to clarify samples of insoluble cellular debris. Concentrations of soluble proteins in samples of clarified supernatants were determined using Bradford assays. Samples were heat denatured at 100°C for 5 minutes in protein denaturing sample buffer. Levels of glucocorticoid receptor α protein expression was examined in 50 μg aliquots of denatured protein samples that were subjected to electrophoretic separation through 10% SDS-polyacrylamide gels followed by electrotransfer onto Immobilon-P membrane (Millipore) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH 11) containing 10% methanol. Primary glucocorticoid receptor antibodies (Santa Cruz Biotechnology, sc-1002) and secondary goat anti-rabbit IgG conjugated to

horse radish peroxidase (HRP) (Santa Cruz Biotechnology, sc-2004) were diluted at 1:2000 and 1:5000, respectively, in Tris buffered saline (20 mM Tris-HCl , pH 7.6; 137 mM NaCl; 0.1% Triton X-100) containing 5% non-fat milk (w/v) to immunoblot proteins on western membranes. Immunocomplexes with HRP activity on membranes were developed using enhanced chemiluminescent reagent (Pierce) as substrate and visualized by exposure to x-ray film. Membranes were blotted with primary monoclonal anti-α-tubulin (Sigma, T5168) to control for equal loading of protein samples on gels and transfer onto membranes.

Real-time PCR for GR exon 1A3-2, 1B-2, 1C-2, 8-9α and 8-9β transcripts: Cells were plated at a concentration of $1x10^7$ cells/ml in complete media with or without drug treatment as indicated and incubated at 37°C for four hours. Total RNA was obtained using Ultraspec (Biotecx, Houston TX) trizol reagent per the manufacturer's protocol. Reverse transcription of 2-5 micrograms of total RNA was carried out with the Superscript III reverse transcription kit (Invitrogen, Carlsbad California) primed with random hexomers. An assay for the GR exon 8-9α transcript was devised using primer3 software available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. The forward and reverse flanking oligonucleotide primers were 5'-AGCCATTGTCAAGAGGGAAG-3' and 5'-TGATTGGTGATGATTTCAGCTA-3', respectively. The FAM/TAMRA Taqman probe located in exon 8 was 5'-TCCAGCCAGAACTGGCAGCG-3'. Each reaction contained 900 nM forward and reverse primer, 50 nM probe, 1X Universal PCR Master Mix (Applied Biosystems, Foster City CA), and 2 µl of cDNA diluted 1:50. Flanking primers and internal FAM/TAMRA-labeled probe oligonucleotides and reaction conditions for the splice sites at GR exons 1A3-2, 1B-2 and 1C-2, as well as GR exon 8-9β were those reported by Pedersen and Vedeckis, with the exception that the Taqman probe for the exon 1A3-2 site was [22] 5'-TCAGTGAATATCAACTTCCTTCTCAGACACTTTAATGAA-3' and the reverse primer for the exon 8-9β splice site was TGTGAGATGTGCTTTCTGGTTTTAA [23]. cDNA was diluted 1:50 for measurement of exon 1A3-2, 1B-2, and 1C-2, and diluted 1:5 for measurement of exon 8-9β. Predeveloped Taqman assay reagents (PDAR) for measuring 18S rRNA (Applied Biosystems) was used for normalization. Realtime PCR was carried out on a MX300P instrument (Stratagene, La Jolla,CA) Transcript abundance relative to control samples was calculated using the $2^{-\Delta\Delta Ct}$ method. We established that the slopes of the curves for the amplification of GRα and rRNA did not vary by more than 10%. All oligonucleotides were purchased from IDT.

Apoptosis assays: One million cells were incubated in duplicate in 48 well plates with or without drug treatment as indicated for 48 hours in 1 mL of complete media. Cell were transferred to polypropylene Falcon FACS tubes, incubated for 10 minutes at 37°C with Hoechst 33342 (Molecular Probes, Eugene OR) at a final concentration of 0.25 µg/mL. Cells were then stored on ice until analysis on a MoFlo cytometer using a 450 nm bandpass filter. In some cases, apoptosis was detected as membrane depolarization with

dihexyloxacarbocyanine ($DiOC_6(3)$) from Molecular Probes (Eugene, OR) at a concentration of 400 nM. $DiOC_6(3)$ stained samples were incubated for 30 minutes at 37°C and stored on ice until analysis on a FACScan (Becton Dickenson, Franklin Lakes, NJ). Results obtained from Hoechst 33342 and $DiOC_6(3)$ staining were validated with Annexin-V/PI staining per the manufacturer's protocol (BD, Franklin Lakes, NJ). FACS data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR) by gating for the apoptotic population. The level of apoptosis detected in B-CLL cultures did not differ by more than 10% when measured by Hoechst 33342 or DiOC6(3).

Statistical Analysis: Statistical analysis and plotting was performed using Prism version 4 (GraphPad Software, San Diego CA).

Results

Rolipram augments GRα transcript and protein levels in B-CLL cells in a time and dose-dependent manner. Given prior reports that cAMP analogues augment GR levels in a subset of cell types, we used comparative quantification real-time PCR to determine whether treatment of B-CLL cells with a PDE4 inhibitor altered expression of GRα transcript. In leukemic cells from eight patients, treatment of B-CLL cells with the PDE4 inhibitor rolipram (20 μM) augmented GRα transcript levels in a time and dose-dependent manner. GRα transcript levels rose over the first four hours to a mean of 4.8 +/- 0.2-fold above baseline and maintained such a fourfold increase for at least 24 hours (Figure 1A). While comparable augmentation of GR transcript levels was observed at rolipram doses ranging from 1 to 20 μM, significant augmentation was not observed at 0.1 μM rolipram, a concentration at or below the EC_{50} of rolipram for inhibition of TNF secretion (Figure 1B) [25]. Addition of the adenylate cyclase stimulator forskolin did not significantly augment GRα transcript in B-CLL cells, either when used alone or in combination with rolipram, a finding in keeping with prior studies demonstrating that rolipram activates PKA in B-CLL in the absence of exogenous adenylate cyclase activation (data not shown). Western analysis of rolipram-treated B-CLL cells demonstrated that PDE4-inhibitor-induced GR transcript up-regulation was associated with an increase in GR protein at six hours (Figure 1C).

cAMP-mediated augmentation of GR transcript levels has been variably attributed to increased GR half-life (in rat hepatoma cells) or GR transcription (in HeLa cells) [16,17] To establish whether the increased levels of GR transcript observed in rolipram-treated B-CLL cells were the result of altered transcript half-life, we treated B-CLL cells with vehicle alone (DMSO) or rolipram (20 μM) for four hours, followed by treatment with the RNA

polymerase inhibitor actinomycin D (10 μg/mL) for varying periods of time. Assessment of GRα transcript levels following such actinomycin D treatment revealed that the half-life of GRα transcript was not altered by rolipram treatment ($p = 0.88$, Figure 2), suggesting that in B-CLL cells, cAMP-mediated augmentation of GRα transcript occurs by a transcriptional mechanism.

Rolipram-mediated GRα transcript up-regulation is not observed in a range of normal hematopoietic cell types. In order to establish the specificity of PDE4 inhibitor-mediated GR transcript up-regulation, we carried out real-time PCR analyses in a range of normal hematopoietic cells. Rolipram treatment did not augment GRα transcript levels in unpurified human mononuclear cells or in purified purified populations of human T cells, B cells, neutrophils and monocytes (Figure 3). In the absence of basal adenylate cyclase activity, PDE4 inhibitors may be relatively ineffective in activating cAMP-mediated signal transduction. However, forskolin (40 μM), either alone or in combination with rolipram, did not induce GR transcript up-regulation in these normal hematopoietic cell populations (data not shown). As it possible that a PDE family other than PDE4 might regulate GR levels in these cell populations, we examined whether addition of IBMX (50 μg/mL), a competitive inhibitor of nine of the eleven currently known PDE families, led to an increase in GR transcript. No augmentation of GR transcript levels was observed in unpurified human mononuclear cells (a predominantly T cell preparation) or in purified populations of human monocytes (data not shown). To establish whether the observed effect of PDE4 inhibitors on B-CLL GRα transcript levels was simply a function of lymphoid transformation, we examined primary leukemic cells from a patient with T-CLL and a patient with Sezary syndrome. In neither case was rolipram-induced augmentation of GRα transcript levels observed (Figure 3).

Roflumilast and cilomilast augment glucocorticoid-mediated apoptosis and GRα transcript levels. To determine whether the alterations in GRα transcript observed following rolipram treatment of B-CLL cells are shared by other structurally distinct PDE4 inhibitors, we examined cilomilast and roflumilast, two PDE4 inhibitors that have been utilized in clinical trials testing the activity of PDE4 inhibitors in asthma and chronic obstructive pulmonary disease (COPD) (Figure 4A) [26 27]. Consistent with the hypothesis that PDE4 is in fact the rolipram target that results in augmented GRα transcript levels, both cilomilast and roflumilast increased GRα transcript levels in B-CLL cells (Figure 4B). As previously observed with rolipram, both cilomilast (20 μM) and roflumilast (0.25 μM) augmented the efficacy with which glucocorticoids induce apoptosis in B-CLL (Figure 4C). In pooled data from five patients, a significant effect of combined PDE4 inhibitor/glucocorticoid therapy on levels of apoptosis was observed when compared with vehicle only, PDE4 inhibitor alone or glucocorticoid alone ($p < .001$). When the individual data from these patients is examined (Table 1), it is

apparent that the sensitivity of leukemic cells to apoptosis induced by PDE4 inhibitors, glucocorticoids and combined treatment varied significantly among different patients.

The synergistic apoptotic effects of combined PDE4 inhibitor/glucocorticoid treatment can be observed following drug treatment for as little as two hours. In order to determine whether the synergistic effect of combined glucocorticoid/PDE4 inhibitor therapy occurs after a relatively brief exposure to these two classes of compounds, CLL cells were treated with vehicle alone, rolipram (20 μM), dexamethasone (1 μM) or the combination of rolipram and dexamethasone for varying periods of time, followed by washing and completion of cell culture for 48 hours. Combined rolipram/glucocorticoid therapy for as little as two hours augmented apoptosis relative to treatment with either drug alone (Figure 4D). Treatment for eight hours with the drug combination resulted in a level of apoptosis that approached that observed following a full 48 hours of combined drug treatment. Although our studies did not address how much of the compounds remain associated with the leukemic cells after washing, these results suggest that PDE4 inhibitors potentiate glucocorticoid-mediated apoptosis in a relatively short period of time.

PDE4 inhibitors variably augment different classes of GRα transcript. Transcription of the glucocorticoid receptor gene is regulated by three promoters, 1A, 1B, and 1C [28]. Prior studies in the human B cell line IM-9 have demonstrated that under basal conditions, roughly 1%, 32% and 66% of GRα transcripts are derived from promoters 1A, 1B and 1C, respectively [24]. Using previously validated real-time PCR assays that detect splicing of exons 1A3, 1B and 1C to exon 2, we examined leukemic cells from six B-CLL patients for the effect of rolipram treatment on levels of GRα transcripts derived from these three promoters. Rolipram (20 μM) augmented GR transcripts derived from each of the three promoters: exon 1A3 (22.2 +/- 7.4), exon 1B (3.6 +/- 0.5) and exon 1C (7.1 +/- 0.9). The up-regulation observed for transcripts containing exon 1A3 was significantly higher than that observed for transcripts containing exon 1B (3.6 +/- 0.5) (Wilcoxon signed rank test for paired comparisons $p < .01$; Figure 5A).

As GRβ has been reported to suppress the stimulation of GRα-induced transactivation by synthetic glucocorticoids and may correlate with sensitivity to GC-induced apoptosis, we examined GRβ regulation by PDE4 inhibitors in B-CLL as well [29,30]. Treatment with rolipram augmented GRβ transcript levels seven-fold relative to levels observed in untreated CLL cells (Figure 5A). Basal levels of GRβ in B-CLL cells appear to be far lower than those of GRα, as the real-time PCR threshold cycle numbers we observed for GRβ were 10 cycles greater than those for GRα despite comparable efficiency of amplification. These results are similar to the 1000-fold lower GRβ level reported by Vedeckis and colleagues using the same oligonucleotide primers in a

quantitative real-time PCR study of basal and glucocorticoid-treated GRα and β transcript levels in the EBV-transformed B cell-line IM-9 [24].

PDE4 inhibitors abrogate the ability of dexamethasone to reduce B-CLL GRα transcript levels. Exposure to glucocorticoids regulates intracellular GR levels, with resultant down-regulation of GR in most cell lineages, including B cells and B lineage cell lines, but with up-regulation of GR in thymocytes and T-ALL-derived cell lines [24]. Using a tetracycline-regulated GR promoter transfected into a cell-line lacking functional GR, the glucocorticoid-induced autoinduction of GR expression in human T cell lines has been linked to their increased sensitivity to glucocorticoid-mediated apoptosis [31]. We therefore sought to determine whether in CLL cells, co-treatment with PDE4 inhibitors would abrogate glucocorticoid-mediated attenuation of GR transcript levels. As expected, dexamethasone reduced GR transcript levels in CLL cells in a dose-dependent manner such that following treatment for six hours with 1 μM dexamethasone, GR transcript levels were one-third of those observed in untreated cells (Figure 5B). In contrast, co-treatment of CLL cells for six hours with 20 μM rolipram and varying doses of dexamethasone resulted in GR transcript levels above basal levels, even at 1 μM dexamethasone (Figure 5B). These results suggest that PDE4 inhibitors may augment glucocorticoid-mediated apoptosis in B-CLL cells as a result of their ability to block the normal down-regulation of GR transcript levels in glucocorticoid-treated cells.

Discussion

This study demonstrates that treatment with several structurally distinct PDE4 inhibitors augments GR transcript levels in CLL cells but not in normal circulating hematopoietic cells. As co-treatment with PDE4 inhibitors and glucocorticoids also induces apoptosis in B-CLL cells to levels higher than that observed with either agent alone, these results suggest that the simultaneous use of these two classes of drug might be relatively selectively toxic to CLL cells [22]. While it is experimentally difficult to determine whether the alterations in GR expression account for the augmented apoptosis observed when these drugs are combined, a variety of prior studies have demonstrated that levels of GR can play an important role in determining the outcome of glucocorticoid treatment. In cell lines expressing different levels of GR, the magnitude of transcriptional responses to glucocorticoids are roughly proportional to the number of receptors per cell [32]. Thymocytes from transgenic mice carrying two extra copies of the GR show enhanced sensitivity to glucocorticoid-mediated apoptosis [33].

Consistent with actinomycin D experiments demonstrating that PDE4 inhibitors do not substantially alter GR transcript half-life, we find that rolipram augments GR transcripts derived from different promoters to varying

degrees in CLL cells, suggesting a transcriptional mechanism for the observed increase in GR transcript levels. GR transcription in human lymphoid cells is regulated by at least three promoters (A-C), although the open reading frame of the GR gene, which begins in exon 2, is not altered by promoter usage [28]. A quantitative analysis in the IM-9 human B cell line revealed that in such cells, promoters 1A, 1B and 1C accounted for 1%, 30% and 70% of all GR□ transcripts, respectively [24]. Alternative splicing of transcripts derived from the most 5' promoter, 1A, results in three types of transcripts: 1A1, 1A2 and 1A3, the last of which was the most abundant [28]. While a prior study in HeLa cells has suggested cAMP-mediated regulation of the most 3' promoter, 1C, the effects of cAMP signaling on GR promoters 1A and 1B have not been reported [17]. Transcripts containing 1B and 1C appear to be relatively ubiquitously expressed, while expression of exon 1A3-containing transcripts is particularly high in cell lines of hematopoietic lineage [28]. In B-CLL cells, treatment with PDE4 inhibitors augments 1A3 transcripts to a greater degree than other GR transcripts (1A3 > 1B; $p < .01$). It is of interest that basal levels of GR transcripts containing exon 1A correlate with sensitivity to glucocorticoid-induced apoptosis, as expression of this form of GR transcript is particularly high in thymocytes and T lymphoblastoid cell lines [34]. While the functional significance of the fact that PDE4 inhibitors preferentially induce expression of exon 1A3-containing GR transcripts in B-CLL cells remains unknown, it is possible that varying 5' UTRs could alter either GR mRNA translation start site or translation efficiency. Two alternative translation initiation sites in exon two give rise to A and B forms of the GR. GRα B has twice the biologic activity of GRα A in gene expression studies, and different tissue types have differing ratios of GRα A and GRα B [35,36].

Among all the primary hematopoietic cell populations tested, augmentation of GR transcript levels by PDE4 inhibitors is unique to B-CLL cells. This finding is in keeping with a prior study in which, among a variety of circulating hematopoietic cells examined, PDE4-inhibitor-induced EPAC activation was found only in B-CLL cells [21]. The explanation for the unique impact of PDE4 inhibitor treatment on signaling in B-CLL cells remains unknown. Although we do not observe rolipram-induced up-regulation of GR transcript in primary circulating B cell samples, it is still possible that comparable responses might be observed in a normal B cell population that is not well represented among circulating B cells. As PDE4 inhibitors have potent effects on a variety of primary circulating hematopoietic cells, particularly T cells and monocytes, it is clearly not the case that the selective augmentation of GR transcript observed in B-CLL cells is due to the fact that PDE4 inhibitors initiate cAMP-mediated signaling only in B-CLL cells. Instead, the selectivity of the effects observed in CLL cells may be due to the magnitude or kinetics of the cAMP response, the effector proteins activated (i.e. type 1 vs. type 2 PKA, EPAC), or, perhaps most likely, cell-type-specific signaling induced by cAMP effector protein activation in B-CLL cells.

Glucocorticoids regulate expression of glucocorticoid receptor. In lymphoid cell subsets that are particularly sensitive to glucocorticoid-mediated apoptosis, glucocorticoids augment GR transcript levels [Pedersen, 2004 #7756]. In other cell lineages, including B lineage cells, glucocorticoids reduce GRα transcript levels [Pedersen, 2003 #7755]. Consistent with this literature, we find that treatment of B-CLL cells with dexamethasone reduces GRα transcript levels in a dose-dependent manner. In contrast, treatment with the combination of dexamethasone and a PDE4 inhibitor augmented GRα transcript levels. While treatment with both drugs resulted in augmentation of GRα transcript intermediate between those observed with treatment with either drug alone, even at the highest dose of dexamethasone used (1 μM), co-treatment resulted in an increment rather than a decrement in GRα transcript levels. These results suggest that PDE4 inhibitors may increase glucocorticoid-mediated apoptosis in B-CLL cells because they counteract the normal dampening of glucocorticoid-mediated signaling that occurs in B lineage cells as a result of glucocorticoid-induced down-regulation of GRα levels.

High dose glucocorticoid therapy can lead to clinical responses in a subset of patients with treatment refractory B-CLL [3 4]. The experiments described in this study suggest that addition of a PDE4 inhibitor to such therapy might fairly selectively augment apoptosis in B-CLL cells as a result of a reduction in glucocorticoid-mediated down-regulation of GR transcript levels. Our observation that treatment with PDE4 inhibitors for as few as four hours augments glucocorticoid-induced killing of B-CLL cells augers well for the potential clinical applicability of PDE4 inhibitor/glucocorticoid therapy for B cell malignancies, as it is likely that therapeutically effective serum levels of PDE4 inhibitors could be safely maintained for such a period of time. Our future studies will focus on the mechanisms underlying the selectivity with which PDE4 inhibitors alter cAMP metabolism in CLL cells.

Figure Legends

Figure 1: GRα expression is up-regulated in CLL cells following treatment with the PDE4 inhibitor rolipram. (A) CLL cells were treated for the indicated lengths of time with rolipram (20 μM), followed by RNA isolation, cDNA synthesis and real-time PCR for GRα using oligonucleotides that spanned exons 8 and 9α. Each point represents the fold increase in GRα transcript levels of an individual patient sample relative to the same patient's CLL cells treated with vehicle (DMSO) alone. The mean fold increase in transcript level is denoted with a horizontal line. (B) CLL cells from an individual patient were treated for four hours with DMSO or rolipram at the indicated dosage (μM), followed by RNA isolation and real-time analysis for relative GRα transcript levels. The data are representative of one of two similar experiments. (C) CLL cells were treated with DMSO alone (CT) or rolipram (20 μM) for 30 minutes (30'), one hour (1h) or six hours (6h), followed by lysis, protein quantification and immunoblot analysis for GRα protein expression (GR). Alpha-tubulin (Tub) was also assessed by immunoblot analysis as an internal loading control.

Figure 2: PDE4 inhibitor-induced GRα transcript up-regulation is not due to altered transcript half-life. CLL cells were treated for four hours with vehicle alone (DMSO) or rolipram (20 μM), followed by treatment with the RNA polymerase inhibitor actinomycin D (10 μg/mL) for the indicated period of time. After RNA isolation and cDNA synthesis, samples were analyzed for relative GRα transcript levels by real-time PCR. All GR transcript levels were normalized to that observed in DMSO-treated cells at time 0. A non-linear curve fit for one phase exponential decay revealed no significant difference in the rates of reduction in GRα transcript levels ($p = 0.88$). The experiment is representative of two experiments performed.

Figure 3: Among circulating hematopoietic lineage cells tested, PDE4-inhibitor-induced augmentation of GRα transcript levels is specific to B-CLL. B-CLL cells, T-CLL cells, Sezary cells, human peripheral blood mononuclear cells (PBMC), T cells, B cells, neutrophils (PMNs) or monocytes were incubated for four hours in vehicle alone (DMSO) or rolipram (20 μM) as indicated. RNA was isolated, cDNA synthesized, and relative levels of GRα transcript assessed by real-time PCR. The data is representative of at least two samples tested for all cell populations except T-CLL, where only one patient sample was available.

Figure 4: Effect of several structurally distinct PDE4 inhibitors on glucocorticoid-mediated apoptosis and GRα transcript levels. (A) Molecular structures of cilomilast (1), roflumilast (2), and rolipram (3). (B) CLL cells were treated for four hours with vehicle alone (DMSO) or the indicated concentration of PDE4 inhibitor, followed by evaluation of GRα transcript level by real-time PCR. The data are representative of two patients

tested. (C) CLL cells were treated for 48 hours with PDE4 inhibitors alone (-7) or in the presence of increasing dosages of dexamethasone, as indicated, followed by assessment for apoptosis. The PDE4 inhibitors cilomilast (1127), roflumilast (2028) and rolipram were added at the concentrations indicated in the sidebar. The data shown are the mean and SEM of five patients tested. (D) CLL cells were treated for 0, 4, 8, 24 or 48 hours with vehicle alone, rolipram (20 μM), dexamethasone (1 μM), or the combination of rolipram and dexamethasone. For all cell samples treated with drugs for less than 48 hours, the drugs were removed by washing, followed by completion of culture in media alone until 48 hours had elapsed.

Figure 5: Panel A: Treatment of B-CLL cells with the PDE4 inhibitor rolipram alters relative expression of GR transcripts containing exons 1A, 1B, 1C, 8/9α and 8/9β. B-CLL cells from six patients were treated with rolipram (20 μM) for four hours, followed by RNA isolation, cDNA synthesis and real-time PCR for GRα □□□□□β transcripts using oligonucleotides that spanned exons 1A3/2 (1A), 1B/2 (1B), 1C/2 (1C), or 8/9α (alpha). The rolipram-induced augmentation of GR transcripts containing exon 1A3 sequence was significantly greater than that of transcripts containing exon 1B (Wilcoxon signed rank test for paired comparisons $p < .01$). A comparable analysis was also carried out for transcripts containing exon 8/9β. □□□□ but here only three patient samples were analyzed. **Panel B: Co-treatment with a PDE4 inhibitor and dexamethasone maintains GRα transcript levels at greater than basal levels despite dexamethasone-induced down-regulation of GRα expression.** B-CLL cells were treated with varying dosages of dexamethasone as indicated for four hours in the presence or absence of rolipram (20 μM), followed by RNA isolation, cDNA synthesis and real-time PCR for GRα using oligonucleotides that spanned exons 8 and 9α. All GR transcript levels were normalized to that observed in DMSO-treated cells.

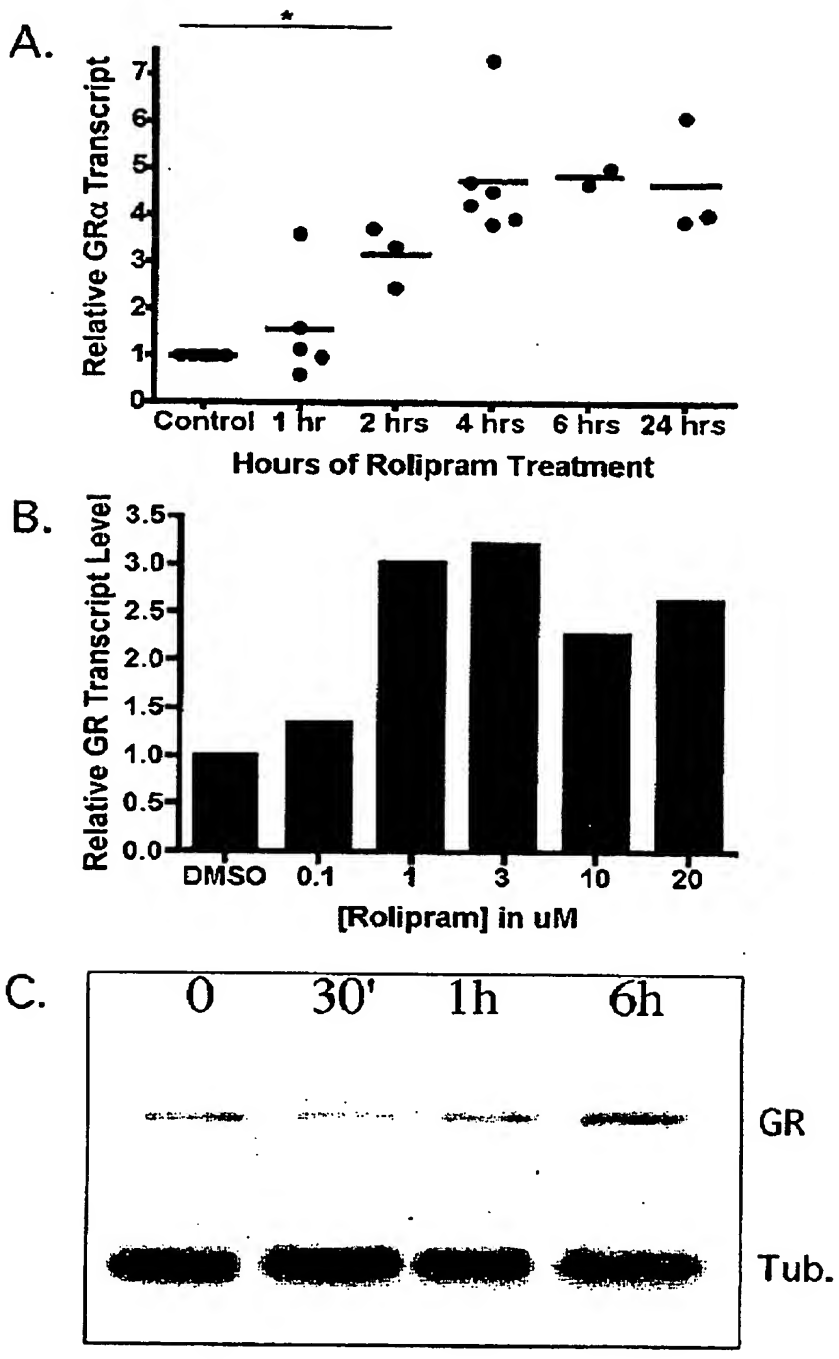

A.
*
Relative GRα Transcript
0
1
2
3
4
5
6
7
Control
1 hr
2 hrs
4 hrs
6 hrs
24 hrs
Hours of Rolipram Treatment
B.
Relative GR Transcript Level
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
DMSO
0.1
1
3
10
20
[Rolipram] in uM
C.
0
30'
1h
6h
GR
Tub.

Figure 1.

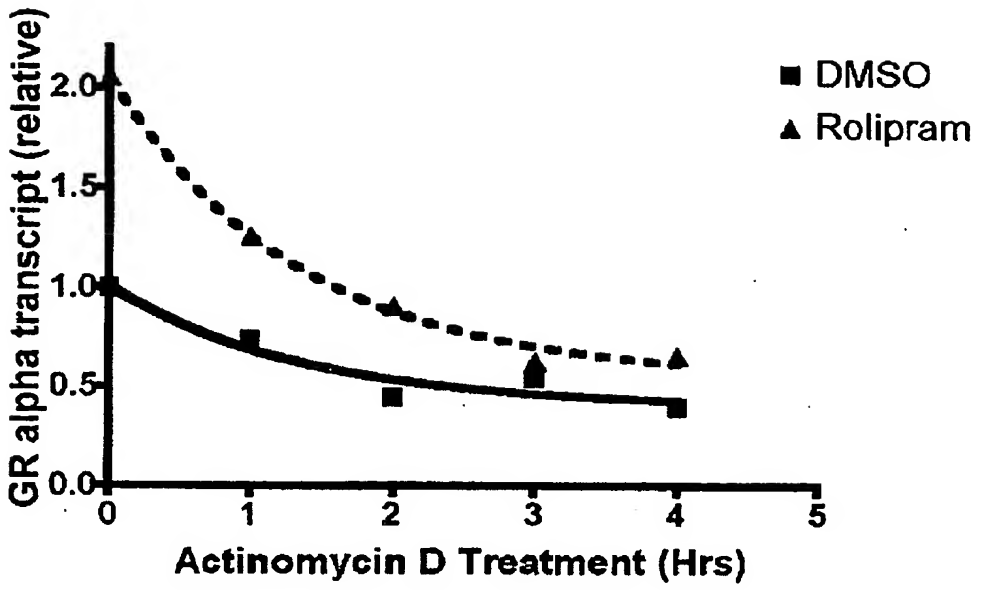

GR alpha transcript (relative)
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Actinomycin D Treatment (Hrs)
DMSO
Rolipram

Figure 2.

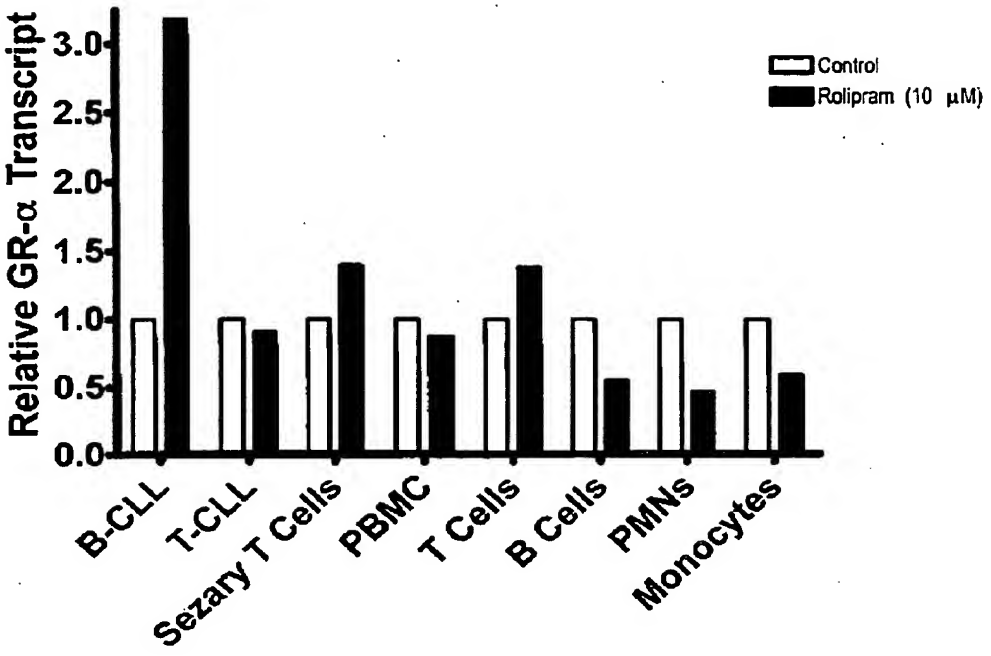

Relative GR-α Transcript
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Rolipram (10 μM)
B-CLL
T-CLL
Sezary T Cells
PBMC
T Cells
B Cells
PMNs
Monocytes

Figure 3.

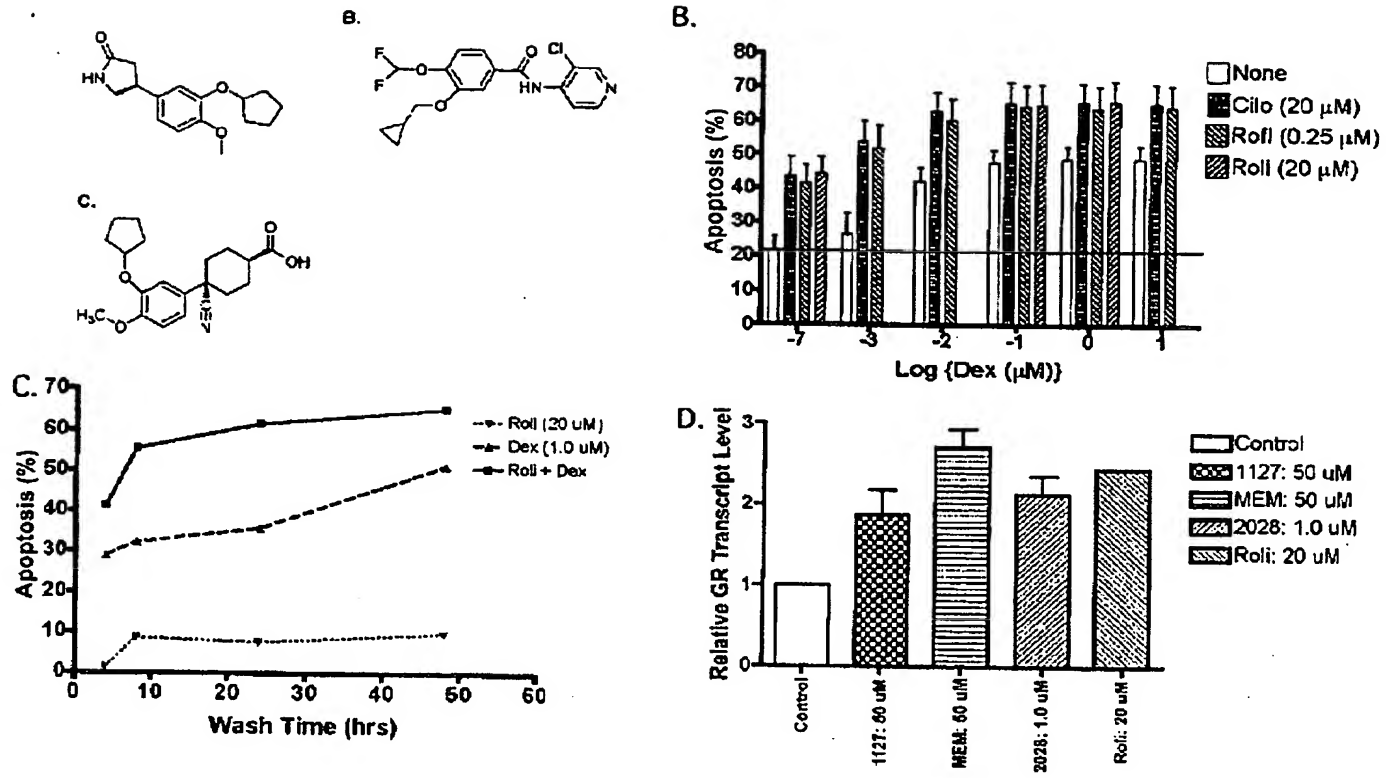
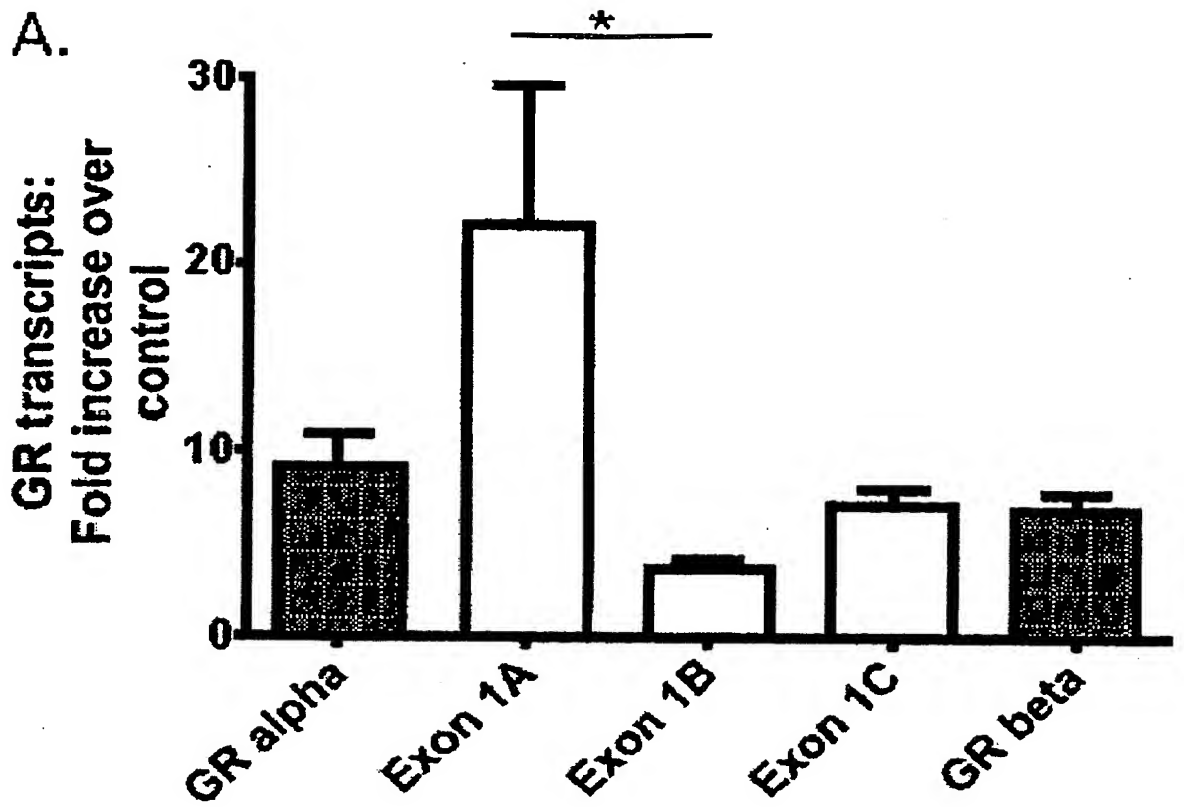

B.
C.
C. 70
Apoptosis (%)
Wash Time (hrs)
Roll (20 uM)
Dex (1.0 uM)
Roll + Dex
B.
Apoptosis (%)
Log {Dex (μM)}
None
Cilo (20 μM)
Rofl (0.25 μM)
Roll (20 μM)
D.
Relative GR Transcript Level
Control
1127: 50 uM
MEM: 50 uM
2028: 1.0 uM
Roli: 20 uM

Figure 4


A.
*
GR transcripts: Fold increase over control
30
20
10
0
GR alpha
Exon 1A
Exon 1B
Exon 1C
GR beta

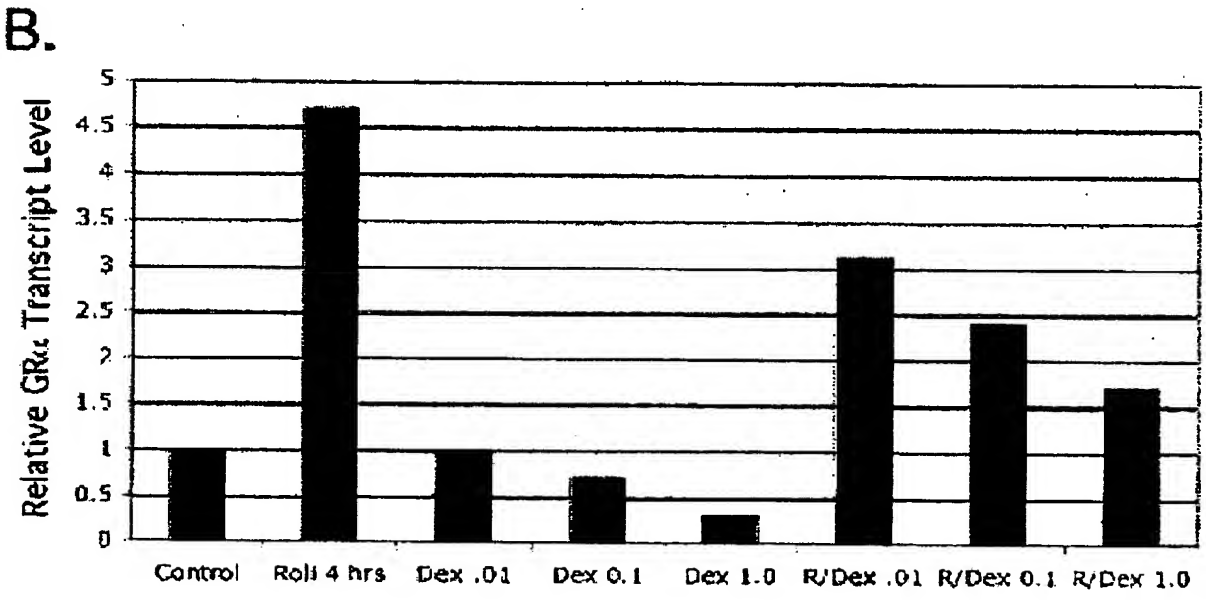

B.
Relative GRα Transcript Level
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Roli 4 hrs
Dex .01
Dex 0.1
Dex 1.0
R/Dex .01
R/Dex 0.1
R/Dex 1.0

Figure 5

References

1. Sawitsky A, Rai KR, Glidewell O, Silver RT. Comparison of daily versus intermittent chlorambucil and prednisone therapy in the treatment of patients with chronic lymphocytic leukemia. Blood. 1977;50:1049-1059.
2. Han T, Ezdinli EZ, Shimaoka K, Desai DV. Chlorambucil vs. combined chlorambucil-corticosteroid therapy in chronic lymphocytic leukemia. Cancer. 1973;31:502-508.
3. Molica S. High-dose dexamethasone in refractory B-cell chronic lymphocytic leukemia patients. Am J Hematol. 1994;47:334.
4. Thornton PD, Hamblin M, Treleaven JG, Matutes E, Lakhani AK, Catovsky D. High dose methyl prednisolone in refractory chronic lymphocytic leukaemia. Leuk Lymphoma. 1999;34:167-170.
5. Moalli PA, Pillay S, Weiner D, Leikin R, Rosen ST. A mechanism of resistance to glucocorticoids in multiple myeloma: transient expression of a truncated glucocorticoid receptor mRNA. Blood. 1992;79:213-222.
6. Irving JA, Minto L, Bailey S, Hall AG. Loss of heterozygosity and somatic mutations of the glucocorticoid receptor gene are rarely found at relapse in pediatric acute lymphoblastic leukemia but may occur in a subpopulation early in the disease course. Cancer Res. 2005;65:9712-9718.
7. de Lange P, Segeren CM, Koper JW, et al. Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. Cancer Res. 2001;61:3937-3941.
8. Rabindran SK, Danielsen M, Stallcup MR. Glucocorticoid-resistant lymphoma cell variants that contain functional glucocorticoid receptors. Mol Cell Biol. 1987;7:4211-4217.
9. Zawydiwski R, Harmon JM, Thompson EB. Glucocorticoid-resistant human acute lymphoblastic leukemic cell line with functional receptor. Cancer Res. 1983;43:3865-3873.
10. Soufi M, Kaiser U, Schneider A, Beato M, Westphal HM. The DNA and steroid binding domains of the glucocorticoid receptor are not altered in mononuclear cells of treated CLL patients. Exp Clin Endocrinol Diabetes. 1995;103:175-183.
11. Gruol DJ, Campbell NF, Bourgeois S. Cyclic AMP-dependent protein kinase promotes glucocorticoid receptor function. J Biol Chem. 1986;261:4909-4914.
12. Gruol DJ, Altschmied J. Synergistic induction of apoptosis with glucocorticoids and 3',5'-cyclic adenosine monophosphate reveals agonist activity by RU 486. Mol Endocrinol. 1993;7:104-113.
13. Kiefer J, Okret S, Jondal M, McConkey DJ. Functional glucocorticoid receptor expression is required for cAMP-mediated apoptosis in a human leukemic T cell line. J Immunol. 1995;155:4525-4528.

14. Doucas V, Shi Y, Miyamoto S, West A, Verma I, Evans RM. Cytoplasmic catalytic subunit of protein kinase A mediates cross-repression by NF-kB and the glucocorticoid receptor. Proc Natl Acad Sci U S A. 2000;97:11893-11898.

15. Gruol DJ, Rajah FM, Bourgeois S. Cyclic AMP-dependent protein kinase modulation of the glucocorticoid-induced cytolytic response in murine T-lymphoma cells. Mol Endocrinol. 1989;3:2119-2127.

16. Dong Y, Aronsson M, Gustafsson JA, Okret S. The mechanism of cAMP-induced glucocorticoid receptor expression. Correlation to cellular glucocorticoid response. J Biol Chem. 1989;264:13679-13683.

17. Penuelas I, Encio IJ, Lopez-Moratalla N, Santiago E. cAMP activates transcription of the human glucocorticoid receptor gene promoter. J Steroid Biochem Mol Biol. 1998;67:89-94.

18. Lerner A, Epstein PM. Cyclic nucleotide phosphodiesterases as targets for treatment of haematological malignancies. Biochem J. 2006;393:21-41.

19. Kim DH, Lerner A. Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. Blood. 1998;92:2484-2494.

20. Moon EY, Lerner A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. Blood. 2003;101:4122-4130.

21. Tiwari S, Felekkis K, Moon EY, Flies A, Sherr DH, Lerner A. Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1, but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. Blood. 2004;103:2661-2667.

22. Tiwari S, Dong H, Kim EJ, Weintraub L, Epstein PM, Lerner A. Type 4 cAMP phosphodiesterase (PDE4) inhibitors augment glucocorticoid-mediated apoptosis in B cell chronic lymphocytic leukemia (B-CLL) in the absence of exogenous adenylyl cyclase stimulation. Biochem Pharmacol. 2005;69:473-483.

23. Fan W-T, Koch CA, de Hoog CL, Fam NP, Moran MF. The exchange factor Ras-GRF2 activates Ras-dependent and Rac-dependent mitogen-activated protein kinase pathways. Curr Biol. 1998;8:935-938.

24. Pedersen KB, Vedeckis WV. Quantification and glucocorticoid regulation of glucocorticoid receptor transcripts in two human leukemic cell lines. Biochemistry. 2003;42:10978-10990.

25. Seldon PM, Meja KK, Giembycz MA. Rolipram, salbutamol and prostaglandin E2 suppress TNFalpha release from human monocytes by activating Type II cAMP-dependent protein kinase. Pulm Pharmacol Ther. 2005;18:277-284.

26. Rennard SI, Schachter N, Strek M, Rickard K, Amit O. Cilomilast for COPD: results of a 6-month, placebo-controlled study of a potent, selective inhibitor of phosphodiesterase 4. Chest. 2006;129:56-66.

27. Timmer W, Leclerc V, Birraux G, et al. The new phosphodiesterase 4 inhibitor roflumilast is efficacious in exercise-induced asthma and leads to suppression of LPS-stimulated TNF-alpha ex vivo. J Clin Pharmacol. 2002;42:297-303.

28. Breslin MB, Geng CD, Vedeckis WV. Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. Mol Endocrinol. 2001;15:1381-1395.

29. Fruchter O, Kino T, Zoumakis E, et al. The human glucocorticoid receptor (GR) isoform {beta} differentially suppresses GR{alpha}-induced transactivation stimulated by synthetic glucocorticoids. J Clin Endocrinol Metab. 2005;90:3505-3509.

30. Koga Y, Matsuzaki A, Suminoe A, Hattori H, Kanemitsu S, Hara T. Differential mRNA expression of glucocorticoid receptor alpha and beta is associated with glucocorticoid sensitivity of acute lymphoblastic leukemia in children. Pediatr Blood Cancer. 2005;45:121-127.

31. Ramdas J, Liu W, Harmon JM. Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Res. 1999;59:1378-1385.

32. Vanderbilt JN, Miesfeld R, Maler BA, Yamamoto KR. Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. Mol Endocrinol. 1987;1:68-74.

33. Reichardt HM, Umland T, Bauer A, Kretz O, Schutz G. Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. Mol Cell Biol. 2000;20:9009-9017.

34. Purton JF, Monk JA, Liddicoat DR, et al. Expression of the glucocorticoid receptor from the 1A promoter correlates with T lymphocyte sensitivity to glucocorticoid-induced cell death. J Immunol. 2004;173:3816-3824.

35. Yudt MR, Cidlowski JA. Molecular identification and characterization of a and b forms of the glucocorticoid receptor. Mol Endocrinol. 2001;15:1093-1103.

36. Lu NZ, Cidlowski JA. Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Mol Cell. 2005;18:331-342.

EXHIBIT B

PDE4 inhibitors augment levels of glucocorticoid receptor in B cell chronic lymphocytic leukemia but not in normal circulating hematopoietic cells

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Abstract

Type 4 cAMP phosphodiesterase (PDE4) inhibitors, a class of compounds in clinical development that activate cAMP-mediated signaling by inhibiting cAMP catabolism, offer a feasible means by which to potentiate glucocorticoid-mediated apoptosis in lymphoid malignancies such as B cell chronic lymphocytic leukemia (B-CLL). In this study, we demonstrate that PDE4 inhibitors up-regulate glucocorticoid receptor (GRα) transcript levels in B-CLL cells but not T-CLL cells or Sezary cells or normal circulating T cells, B cells, monocytes or neutrophils. As GRα transcript half-life does not vary in CLL cells treated with rolipram, the four-fold increase in GRα mRNA levels observed within four hours of rolipram treatment appears to result from an increase in GRα transcription. Rolipram treatment increases levels of transcripts derived from the 1A3 promoter to a greater extent than the 1B promoter. Treatment of B-CLL cells with two PDE4 inhibitors currently in clinical trials also augments glucocorticoid receptor transcript levels and glucocorticoid-mediated apoptosis. Washout studies demonstrate that treatment with both drug classes irreversibly augments apoptosis over the same time frame that glucocorticoid receptor up-regulation occurs. While treatment of B-CLL cells with glucocorticoids reduces basal GRα transcript levels in a dose-related manner, co-treatment with rolipram results in GRα transcript levels above baseline that are intermediate between those observed following treatment with either drug alone. Our results suggest that as a result of their unusual sensitivity to PDE4 inhibitor-mediated up-regulation of GRα expression, treatment of B-CLL patients with combined PDE4 inhibitor/glucocorticoid therapy may be of therapeutic benefit in this disease.

Introduction

Glucocorticoids are an important component of standard therapy for several lymphoid malignancies, including multiple myeloma, acute lymphoblastic leukemia and diffuse large B cell lymphoma. As early studies in patients with B cell chronic lymphocytic leukemia (B-CLL) demonstrated that addition of prednisone to chlorambucil augmented response rate but not median survival, glucocorticoids are not generally a standard component of initial therapy for patients with B-CLL [1,2]. Nonetheless, two studies of high dose glucocorticoid therapy have suggested that glucocorticoids can be of clinical benefit to a subset of patients with treatment-refractory B-CLL [3,4]. Despite frequent responses to glucocorticoid treatment, monotherapy with glucocorticoids is not curative in any lymphoid malignancy, but the mechanisms underlying clinical glucocorticoid resistance remain controversial. Structural alterations in the GR are commonly identified in lymphoid cell lines that have been selected for glucocorticoid resistance by prolonged culture in dexamethasone, but comparable alterations in primary malignant lymphoid cells have been only infrequently reported [5-9]. A detailed analysis of treated B-CLL patients failed to identify abnormalities in either the DNA or steroid binding domains of leukemic GRs [10]. Non-structural modifications of glucocorticoid signaling pathways are likely to be important in clinical glucocorticoid resistance and efforts to identify and reverse these modifications may be therapeutically useful [8,9].

cAMP-mediated signaling can favorably alter the apoptotic response to glucocorticoids in specific lymphoid subsets, although the precise molecular explanation for this relationship remains unclear. Seminal early work done by Suzanne Bourgeois and colleagues demonstrated that isolation of Wehi-7 cells, a murine T cell lymphoma line, that were resistant to cAMP-mediated apoptosis due to alterations in protein kinase A made subsequent development of spontaneous glucocorticoid-resistant cells occur at higher frequencies (10^{-7}) than in wildtype cells ($> 10^{-10}$) [11]. Gruol and Altschmied subsequently determined that RU486, ordinarily a GR antagonist for GC-induced lymphoid cytolysis, becomes an agonist in the setting of co-treatment with a cAMP analogue [12]. Conversely, McConkey and colleagues reported that a glucocorticoid receptor (GR) deficient ICR.27 cells, a variant of the CEM T cell lymphoma line, are insensitive to cAMP-induced apoptosis. Transfection of ICR.27 cells with the glucocorticoid receptor restored sensitivity to cAMP-mediated apoptosis [13]. Finally, the catalytic subunit of PKA has been shown to associate with the glucocorticoid receptor [14].

One critical factor that regulates lymphoid sensitivity to glucocorticoids is the level of GR expression. Gruol et al determined that treatment of Wehi-7 cells with cAMP analogues raised glucocorticoid receptor transcript and protein levels [15]. Several mechanisms have been proposed to explain why GR transcript levels rise following treatment of specific cell subsets with agents that augment cAMP signaling. In studies of rat hepatoma cells,

Dong et al reported that treatment with 8-bromo-cAMP increases GR mRNA half-life from 4 hours to 10 hours [16]. As treatment of such cell cultures with inhibitors of protein or mRNA synthesis had no effect on the ability of 8-bromo-cAMP to increase GR transcript levels, Dong et al argue that a principal mechanism by which cAMP signaling augments GR transcript levels must be through GR mRNA stabilization. In contrast, utilizing transfection of GR promoter luciferase constructs into HeLa cells, Penuelas et al determined that treatment with the adenylate cyclase activator forskolin doubled the transcriptional activity of the human GR promoter [17]. After mapping and testing five putative CRE binding sites, the authors demonstrated loss of forskolin inducibility in promoter constructs shorter than 1 kB and the presence of a CRE element which bound CRE in vitro in shift assays. Thus, it appears that in some cell lineages, cAMP-induced augmentation of GR transcript levels is due to augmented transcription rather than mRNA stabilization.

Type 4 cAMP phosphodiesterase (PDE4) inhibitors offer a therapeutically plausible means by which to take advantage of the phenomenon of cAMP-mediated augmentation of glucocorticoid sensitivity in malignant lymphoid cells. The PDE4 family play a key role in catabolizing cAMP in a variety of human hematopoietic cells and PDE4 inhibitors are in late stage clinical studies for a variety of inflammatory illnesses, including asthma and chronic obstructive disease [18]. In prior work, we have determined that inhibition of PDE4, in the absence of addition of exogenous adenylate cyclase activators such as forskolin or beta adrenergic agonists, increases cAMP levels, activates protein kinase A as judged by CREB phosphorylation, and induces apoptosis in primary B-CLL cells, albeit in well less than 100% of cells [19]. Treatment with the prototypic PDE4 inhibitor rolipram induces mitochondrial release of cytochrome c, activation of caspase 9 and 3, and cleavage of PARP in CLL cells [20]. PDE4 inhibitors also activate Rap1 in B-CLL cells due to cAMP-mediated activation of the Rap1 GDP exchange factor EPAC1, but EPAC activation appears to be anti-apoptotic [21]. PDE4 inhibitors thus induce both PKA-mediated pro-apoptotic and EPAC-mediated anti-apoptotic signaling pathways in B-CLL cells, with the PKA-mediated pro-apoptotic pathway having a dominant effect.

PDE4 inhibitors such as rolipram augment hydrocortisone or dexamethasone-induced apoptosis in primary B-CLL cells, as well as transactivation of glucocorticoid response element (GRE)-containing reporter constructs [22]. Both of these effects are reversed by the type I PKA antagonist *Rp*-8Br-cAMPS. The specific mechanism or mechanisms by which PDE4 inhibitors increase glucocorticoid sensitivity in B-CLL cells remain unknown. In this study, we sought to determine whether PDE4 inhibitors alter leukemic expression of glucocorticoid receptor. We find that PDE4 inhibitors augment expression of GRα at a transcriptional level and that among human primary hematopoietic cells, this effect is quite specific to B-CLL.

Methods and Materials

Materials: The following reagents were obtained from commercial sources: R-(-)-Rolipram (Biomol, Plymouth Meeting PA), forskolin (Sigma, St. Louis MO), 1-Methyl-3-isobutylxanthine [IBMX] (Sigma), actinomycin D (Calbiochem, San Diego CA). Cilomilast and roflumilast were obtained from Memory Pharmaceuticals (Montvale, NJ).

Cell Culture and Isolation: Blood samples were obtained in heparinized tubes with IRB-approved consent from flow cytometry-confirmed B-CLL patients that were either untreated or for whom at least 1 month had elapsed since chemotherapy. Patients with active infections or other serious medical conditions were not included in this study. Patients with white blood cell counts of less than 15,000/μl by automated analysis were excluded from this study. Whole blood was layered on Ficoll-Hystopaque (Sigma) and peripheral blood mononuclear cells (PBMC) isolated after centrification. PBMC were washed and resuspended at $1x10^7$ cells/ml in complete media [RPMI-1640 (Mediatech) supplemented with 10% fetal bovine serum (Sigma), 20mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Mediatech)]. PBMC was found to contain >90% B-CLL by FACS without additional purification. Normal B cells, T cells, and monocytes were obtained from anonymous healthy donors (New York Biologics, Southampton NY) and isolated via magnetic negative depletion per the manufacturer's protocol (Miltenyi, Bergisch-Gladbach Germany) from PBMC. Polymorphonuclear cells (PMNs) were obtained by erythrocyte depletion of whole blood via dextran sedimentation followed by removal of PBMC using Ficoll seperation. With the exception of PMNs, which were used immediately after purification, all other primary normal and malignant cell populations were rested overnight at 37°C prior to use.

Western analysis: Following cell culture, cells were collected by centrifugation (400xg; 10 min.), washed once with phosphate buffered saline and lysed in ice-cold 10 mM HEPES-NaOH buffer (pH 7.4) containing 1%TritonX-100, 10% glycerol, 25 mM β-glycerophosphate, 100 mM NaCl, 2mM EDTA, 2mM EGTA, 1mM dithiothriotol, 1 mM vanadate, 1mM phenylmethanesulfonyl fluoride, and 1mM benzamidine. Cell lysates were transferred to 1.5 mL tubes and centrifuged at 14,000 rpm for 30 minutes (5°C) in a microcentrifuge to clarify samples of insoluble cellular debris. Concentrations of soluble proteins in samples of clarified supernatants were determined using Bradford assays. Samples were heat denatured at 100°C for 5 minutes in protein denaturing sample buffer. Levels of glucocorticoid receptor α protein expression was examined in 50 μg aliquots of denatured protein samples that were subjected to electrophoretic separation through 10% SDS-polyacrylamide gels followed by electrotransfer onto Immobilon-P membrane (Millipore) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH 11) containing 10% methanol. Primary glucocorticoid receptor antibodies (Santa Cruz Biotechnology, sc-1002) and secondary goat anti-rabbit IgG conjugated to

horse radish peroxidase (HRP) (Santa Cruz Biotechnology, sc-2004) were diluted at 1:2000 and 1:5000, respectively, in Tris buffered saline (20 mM Tris-HCl , pH 7.6; 137 mM NaCl; 0.1% Triton X-100) containing 5% non-fat milk (w/v) to immunoblot proteins on western membranes. Immunocomplexes with HRP activity on membranes were developed using enhanced chemiluminescent reagent (Pierce) as substrate and visualized by exposure to x-ray film. Membranes were blotted with primary monoclonal anti-α-tubulin (Sigma, T5168) to control for equal loading of protein samples on gels and transfer onto membranes.

Real-time PCR for GR exon 1A3-2, 1B-2, 1C-2, 8-9α and 8-9β transcripts: Cells were plated at a concentration of $1x10^7$ cells/ml in complete media with or without drug treatment as indicated and incubated at 37°C for four hours. Total RNA was obtained using Ultraspec (Biotecx, Houston TX) trizol reagent per the manufacturer's protocol. Reverse transcription of 2-5 micrograms of total RNA was carried out with the Superscript III reverse transcription kit (Invitrogen, Carlsbad California) primed with random hexomers. An assay for the GR exon 8-9α transcript was devised using primer3 software available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. The forward and reverse flanking oligonucleotide primers were 5'-AGCCATTGTCAAGAGGGAAG-3' and 5'-TGATTGGTGATGATTTCAGCTA-3', respectively. The FAM/TAMRA Taqman probe located in exon 8 was 5'-TCCAGCCAGAACTGGCAGCG-3'. Each reaction contained 900 nM forward and reverse primer, 50 nM probe, 1X Universal PCR Master Mix (Applied Biosystems, Foster City CA), and 2 µl of cDNA diluted 1:50. Flanking primers and internal FAM/TAMRA-labeled probe oligonucleotides and reaction conditions for the splice sites at GR exons 1A3-2, 1B-2 and 1C-2, as well as GR exon 8-9β were those reported by Pedersen and Vedeckis, with the exception that the Taqman probe for the exon 1A3-2 site was [22] 5'-TCAGTGAATATCAACTTCCTTCTCAGACACTTTAATGAA-3' and the reverse primer for the exon 8-9β splice site was TGTGAGATGTGCTTTCTGGTTTTAA [23]. cDNA was diluted 1:50 for measurement of exon 1A3-2, 1B-2, and 1C-2, and diluted 1:5 for measurement of exon 8-9β. Predeveloped Taqman assay reagents (PDAR) for measuring 18S rRNA (Applied Biosystems) was used for normalization. Realtime PCR was carried out on a MX300P instrument (Stratagene, La Jolla,CA) Transcript abundance relative to control samples was calculated using the $2^{-\Delta\Delta Ct}$ method. We established that the slopes of the curves for the amplification of GRα and rRNA did not vary by more than 10%. All oligonucleotides were purchased from IDT.

Apoptosis assays: One million cells were incubated in duplicate in 48 well plates with or without drug treatment as indicated for 48 hours in 1 mL of complete media. Cell were transferred to polypropylene Falcon FACS tubes, incubated for 10 minutes at 37°C with Hoechst 33342 (Molecular Probes, Eugene OR) at a final concentration of 0.25 µg/mL. Cells were then stored on ice until analysis on a MoFlo cytometer using a 450 nm bandpass filter. In some cases, apoptosis was detected as membrane depolarization with

dihexyloxacarbocyanine ($DiOC_6(3)$) from Molecular Probes (Eugene, OR) at a concentration of 400 nM. $DiOC_6(3)$ stained samples were incubated for 30 minutes at 37°C and stored on ice until analysis on a FACScan (Becton Dickenson, Franklin Lakes, NJ). Results obtained from Hoechst 33342 and $DiOC_6(3)$ staining were validated with Annexin-V/PI staining per the manufacturer's protocol (BD, Franklin Lakes, NJ). FACS data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR) by gating for the apoptotic population. The level of apoptosis detected in B-CLL cultures did not differ by more than 10% when measured by Hoechst 33342 or DiOC6(3).

Statistical Analysis: Statistical analysis and plotting was performed using Prism version 4 (GraphPad Software, San Diego CA).

Results

Rolipram augments GRα transcript and protein levels in B-CLL cells in a time and dose-dependent manner. Given prior reports that cAMP analogues augment GR levels in a subset of cell types, we used comparative quantification real-time PCR to determine whether treatment of B-CLL cells with a PDE4 inhibitor altered expression of GRα transcript. In leukemic cells from eight patients, treatment of B-CLL cells with the PDE4 inhibitor rolipram (20 μM) augmented GRα transcript levels in a time and dose-dependent manner. GRα transcript levels rose over the first four hours to a mean of 4.8 +/- 0.2-fold above baseline and maintained such a fourfold increase for at least 24 hours (Figure 1A). While comparable augmentation of GR transcript levels was observed at rolipram doses ranging from 1 to 20 μM, significant augmentation was not observed at 0.1 μM rolipram, a concentration at or below the EC_{50} of rolipram for inhibition of TNF secretion (Figure 1B) [25]. Addition of the adenylate cyclase stimulator forskolin did not significantly augment GRα transcript in B-CLL cells, either when used alone or in combination with rolipram, a finding in keeping with prior studies demonstrating that rolipram activates PKA in B-CLL in the absence of exogenous adenylate cyclase activation (data not shown). Western analysis of rolipram-treated B-CLL cells demonstrated that PDE4-inhibitor-induced GR transcript up-regulation was associated with an increase in GR protein at six hours (Figure 1C).

cAMP-mediated augmentation of GR transcript levels has been variably attributed to increased GR half-life (in rat hepatoma cells) or GR transcription (in HeLa cells) [16,17] To establish whether the increased levels of GR transcript observed in rolipram-treated B-CLL cells were the result of altered transcript half-life, we treated B-CLL cells with vehicle alone (DMSO) or rolipram (20 μM) for four hours, followed by treatment with the RNA

polymerase inhibitor actinomycin D (10 μg/mL) for varying periods of time. Assessment of GRα transcript levels following such actinomycin D treatment revealed that the half-life of GRα transcript was not altered by rolipram treatment ($p = 0.88$, Figure 2), suggesting that in B-CLL cells, cAMP-mediated augmentation of GRα transcript occurs by a transcriptional mechanism.

Rolipram-mediated GRα transcript up-regulation is not observed in a range of normal hematopoietic cell types. In order to establish the specificity of PDE4 inhibitor-mediated GR transcript up-regulation, we carried out real-time PCR analyses in a range of normal hematopoietic cells. Rolipram treatment did not augment GRα transcript levels in unpurified human mononuclear cells or in purified purified populations of human T cells, B cells, neutrophils and monocytes (Figure 3). In the absence of basal adenylate cyclase activity, PDE4 inhibitors may be relatively ineffective in activating cAMP-mediated signal transduction. However, forskolin (40 μM), either alone or in combination with rolipram, did not induce GR transcript up-regulation in these normal hematopoietic cell populations (data not shown). As it possible that a PDE family other than PDE4 might regulate GR levels in these cell populations, we examined whether addition of IBMX (50 μg/mL), a competitive inhibitor of nine of the eleven currently known PDE families, led to an increase in GR transcript. No augmentation of GR transcript levels was observed in unpurified human mononuclear cells (a predominantly T cell preparation) or in purified populations of human monocytes (data not shown). To establish whether the observed effect of PDE4 inhibitors on B-CLL GRα transcript levels was simply a function of lymphoid transformation, we examined primary leukemic cells from a patient with T-CLL and a patient with Sezary syndrome. In neither case was rolipram-induced augmentation of GRα transcript levels observed (Figure 3).

Roflumilast and cilomilast augment glucocorticoid-mediated apoptosis and GRα transcript levels. To determine whether the alterations in GRα transcript observed following rolipram treatment of B-CLL cells are shared by other structurally distinct PDE4 inhibitors, we examined cilomilast and roflumilast, two PDE4 inhibitors that have been utilized in clinical trials testing the activity of PDE4 inhibitors in asthma and chronic obstructive pulmonary disease (COPD) (Figure 4A) [26 27]. Consistent with the hypothesis that PDE4 is in fact the rolipram target that results in augmented GRα transcript levels, both cilomilast and roflumilast increased GRα transcript levels in B-CLL cells (Figure 4B). As previously observed with rolipram, both cilomilast (20 μM) and roflumilast (0.25 μM) augmented the efficacy with which glucocorticoids induce apoptosis in B-CLL (Figure 4C). In pooled data from five patients, a significant effect of combined PDE4 inhibitor/glucocorticoid therapy on levels of apoptosis was observed when compared with vehicle only, PDE4 inhibitor alone or glucocorticoid alone ($p < .001$). When the individual data from these patients is examined (Table 1), it is

apparent that the sensitivity of leukemic cells to apoptosis induced by PDE4 inhibitors, glucocorticoids and combined treatment varied significantly among different patients.

The synergistic apoptotic effects of combined PDE4 inhibitor/glucocorticoid treatment can be observed following drug treatment for as little as two hours. In order to determine whether the synergistic effect of combined glucocorticoid/PDE4 inhibitor therapy occurs after a relatively brief exposure to these two classes of compounds, CLL cells were treated with vehicle alone, rolipram (20 μM), dexamethasone (1 μM) or the combination of rolipram and dexamethasone for varying periods of time, followed by washing and completion of cell culture for 48 hours. Combined rolipram/glucocorticoid therapy for as little as two hours augmented apoptosis relative to treatment with either drug alone (Figure 4D). Treatment for eight hours with the drug combination resulted in a level of apoptosis that approached that observed following a full 48 hours of combined drug treatment. Although our studies did not address how much of the compounds remain associated with the leukemic cells after washing, these results suggest that PDE4 inhibitors potentiate glucocorticoid-mediated apoptosis in a relatively short period of time.

PDE4 inhibitors variably augment different classes of GRα transcript. Transcription of the glucocorticoid receptor gene is regulated by three promoters, 1A, 1B, and 1C [28]. Prior studies in the human B cell line IM-9 have demonstrated that under basal conditions, roughly 1%, 32% and 66% of GRα transcripts are derived from promoters 1A, 1B and 1C, respectively [24]. Using previously validated real-time PCR assays that detect splicing of exons 1A3, 1B and 1C to exon 2, we examined leukemic cells from six B-CLL patients for the effect of rolipram treatment on levels of GRα transcripts derived from these three promoters. Rolipram (20 μM) augmented GR transcripts derived from each of the three promoters: exon 1A3 (22.2 +/- 7.4), exon 1B (3.6 +/- 0.5) and exon 1C (7.1 +/- 0.9). The up-regulation observed for transcripts containing exon 1A3 was significantly higher than that observed for transcripts containing exon 1B (3.6 +/- 0.5) (Wilcoxon signed rank test for paired comparisons $p < .01$; Figure 5A).

As GRβ has been reported to suppress the stimulation of GRα-induced transactivation by synthetic glucocorticoids and may correlate with sensitivity to GC-induced apoptosis, we examined GRβ regulation by PDE4 inhibitors in B-CLL as well [29,30]. Treatment with rolipram augmented GRβ transcript levels seven-fold relative to levels observed in untreated CLL cells (Figure 5A). Basal levels of GRβ in B-CLL cells appear to be far lower than those of GRα, as the real-time PCR threshold cycle numbers we observed for GRβ were 10 cycles greater than those for GRα despite comparable efficiency of amplification. These results are similar to the 1000-fold lower GRβ level reported by Vedeckis and colleagues using the same oligonucleotide primers in a

quantitative real-time PCR study of basal and glucocorticoid-treated GRα and β transcript levels in the EBV-transformed B cell-line IM-9 [24].

PDE4 inhibitors abrogate the ability of dexamethasone to reduce B-CLL GRα transcript levels. Exposure to glucocorticoids regulates intracellular GR levels, with resultant down-regulation of GR in most cell lineages, including B cells and B lineage cell lines, but with up-regulation of GR in thymocytes and T-ALL-derived cell lines [24]. Using a tetracycline-regulated GR promoter transfected into a cell-line lacking functional GR, the glucocorticoid-induced autoinduction of GR expression in human T cell lines has been linked to their increased sensitivity to glucocorticoid-mediated apoptosis [31]. We therefore sought to determine whether in CLL cells, co-treatment with PDE4 inhibitors would abrogate glucocorticoid-mediated attenuation of GR transcript levels. As expected, dexamethasone reduced GR transcript levels in CLL cells in a dose-dependent manner such that following treatment for six hours with 1 μM dexamethasone, GR transcript levels were one-third of those observed in untreated cells (Figure 5B). In contrast, co-treatment of CLL cells for six hours with 20 μM rolipram and varying doses of dexamethasone resulted in GR transcript levels above basal levels, even at 1 μM dexamethasone (Figure 5B). These results suggest that PDE4 inhibitors may augment glucocorticoid-mediated apoptosis in B-CLL cells as a result of their ability to block the normal down-regulation of GR transcript levels in glucocorticoid-treated cells.

Discussion

This study demonstrates that treatment with several structurally distinct PDE4 inhibitors augments GR transcript levels in CLL cells but not in normal circulating hematopoietic cells. As co-treatment with PDE4 inhibitors and glucocorticoids also induces apoptosis in B-CLL cells to levels higher than that observed with either agent alone, these results suggest that the simultaneous use of these two classes of drug might be relatively selectively toxic to CLL cells [22]. While it is experimentally difficult to determine whether the alterations in GR expression account for the augmented apoptosis observed when these drugs are combined, a variety of prior studies have demonstrated that levels of GR can play an important role in determining the outcome of glucocorticoid treatment. In cell lines expressing different levels of GR, the magnitude of transcriptional responses to glucocorticoids are roughly proportional to the number of receptors per cell [32]. Thymocytes from transgenic mice carrying two extra copies of the GR show enhanced sensitivity to glucocorticoid-mediated apoptosis [33].

Consistent with actinomycin D experiments demonstrating that PDE4 inhibitors do not substantially alter GR transcript half-life, we find that rolipram augments GR transcripts derived from different promoters to varying

degrees in CLL cells, suggesting a transcriptional mechanism for the observed increase in GR transcript levels. GR transcription in human lymphoid cells is regulated by at least three promoters (A-C), although the open reading frame of the GR gene, which begins in exon 2, is not altered by promoter usage [28]. A quantitative analysis in the IM-9 human B cell line revealed that in such cells, promoters 1A, 1B and 1C accounted for 1%, 30% and 70% of all GR□ transcripts, respectively [24]. Alternative splicing of transcripts derived from the most 5' promoter, 1A, results in three types of transcripts: 1A1, 1A2 and 1A3, the last of which was the most abundant [28]. While a prior study in HeLa cells has suggested cAMP-mediated regulation of the most 3' promoter, 1C, the effects of cAMP signaling on GR promoters 1A and 1B have not been reported [17]. Transcripts containing 1B and 1C appear to be relatively ubiquitously expressed, while expression of exon 1A3-containing transcripts is particularly high in cell lines of hematopoietic lineage [28]. In B-CLL cells, treatment with PDE4 inhibitors augments 1A3 transcripts to a greater degree than other GR transcripts (1A3 > 1B; $p < .01$). It is of interest that basal levels of GR transcripts containing exon 1A correlate with sensitivity to glucocorticoid-induced apoptosis, as expression of this form of GR transcript is particularly high in thymocytes and T lymphoblastoid cell lines [34]. While the functional significance of the fact that PDE4 inhibitors preferentially induce expression of exon 1A3-containing GR transcripts in B-CLL cells remains unknown, it is possible that varying 5' UTRs could alter either GR mRNA translation start site or translation efficiency. Two alternative translation initiation sites in exon two give rise to A and B forms of the GR. GRα B has twice the biologic activity of GRα A in gene expression studies, and different tissue types have differing ratios of GRα A and GRα B [35,36].

Among all the primary hematopoietic cell populations tested, augmentation of GR transcript levels by PDE4 inhibitors is unique to B-CLL cells. This finding is in keeping with a prior study in which, among a variety of circulating hematopoietic cells examined, PDE4-inhibitor-induced EPAC activation was found only in B-CLL cells [21]. The explanation for the unique impact of PDE4 inhibitor treatment on signaling in B-CLL cells remains unknown. Although we do not observe rolipram-induced up-regulation of GR transcript in primary circulating B cell samples, it is still possible that comparable responses might be observed in a normal B cell population that is not well represented among circulating B cells. As PDE4 inhibitors have potent effects on a variety of primary circulating hematopoietic cells, particularly T cells and monocytes, it is clearly not the case that the selective augmentation of GR transcript observed in B-CLL cells is due to the fact that PDE4 inhibitors initiate cAMP-mediated signaling only in B-CLL cells. Instead, the selectivity of the effects observed in CLL cells may be due to the magnitude or kinetics of the cAMP response, the effector proteins activated (i.e. type 1 vs. type 2 PKA, EPAC), or, perhaps most likely, cell-type-specific signaling induced by cAMP effector protein activation in B-CLL cells.

Glucocorticoids regulate expression of glucocorticoid receptor. In lymphoid cell subsets that are particularly sensitive to glucocorticoid-mediated apoptosis, glucocorticoids augment GR transcript levels [Pedersen, 2004 #7756]. In other cell lineages, including B lineage cells, glucocorticoids reduce GRα transcript levels [Pedersen, 2003 #7755]. Consistent with this literature, we find that treatment of B-CLL cells with dexamethasone reduces GRα transcript levels in a dose-dependent manner. In contrast, treatment with the combination of dexamethasone and a PDE4 inhibitor augmented GRα transcript levels. While treatment with both drugs resulted in augmentation of GRα transcript intermediate between those observed with treatment with either drug alone, even at the highest dose of dexamethasone used (1 μM), co-treatment resulted in an increment rather than a decrement in GRα transcript levels. These results suggest that PDE4 inhibitors may increase glucocorticoid-mediated apoptosis in B-CLL cells because they counteract the normal dampening of glucocorticoid-mediated signaling that occurs in B lineage cells as a result of glucocorticoid-induced down-regulation of GRα levels.

High dose glucocorticoid therapy can lead to clinical responses in a subset of patients with treatment refractory B-CLL [3 4]. The experiments described in this study suggest that addition of a PDE4 inhibitor to such therapy might fairly selectively augment apoptosis in B-CLL cells as a result of a reduction in glucocorticoid-mediated down-regulation of GR transcript levels. Our observation that treatment with PDE4 inhibitors for as few as four hours augments glucocorticoid-induced killing of B-CLL cells augers well for the potential clinical applicability of PDE4 inhibitor/glucocorticoid therapy for B cell malignancies, as it is likely that therapeutically effective serum levels of PDE4 inhibitors could be safely maintained for such a period of time. Our future studies will focus on the mechanisms underlying the selectivity with which PDE4 inhibitors alter cAMP metabolism in CLL cells.

Figure Legends

Figure 1: GRα expression is up-regulated in CLL cells following treatment with the PDE4 inhibitor rolipram. (A) CLL cells were treated for the indicated lengths of time with rolipram (20 μM), followed by RNA isolation, cDNA synthesis and real-time PCR for GRα using oligonucleotides that spanned exons 8 and 9α. Each point represents the fold increase in GRα transcript levels of an individual patient sample relative to the same patient's CLL cells treated with vehicle (DMSO) alone. The mean fold increase in transcript level is denoted with a horizontal line. (B) CLL cells from an individual patient were treated for four hours with DMSO or rolipram at the indicated dosage (μM), followed by RNA isolation and real-time analysis for relative GRα transcript levels. The data are representative of one of two similar experiments. (C) CLL cells were treated with DMSO alone (CT) or rolipram (20 μM) for 30 minutes (30'), one hour (1h) or six hours (6h), followed by lysis, protein quantification and immunoblot analysis for GRα protein expression (GR). Alpha-tubulin (Tub) was also assessed by immunoblot analysis as an internal loading control.

Figure 2: PDE4 inhibitor-induced GRα transcript up-regulation is not due to altered transcript half-life. CLL cells were treated for four hours with vehicle alone (DMSO) or rolipram (20 μM), followed by treatment with the RNA polymerase inhibitor actinomycin D (10 μg/mL) for the indicated period of time. After RNA isolation and cDNA synthesis, samples were analyzed for relative GRα transcript levels by real-time PCR. All GR transcript levels were normalized to that observed in DMSO-treated cells at time 0. A non-linear curve fit for one phase exponential decay revealed no significant difference in the rates of reduction in GRα transcript levels ($p = 0.88$). The experiment is representative of two experiments performed.

Figure 3: Among circulating hematopoietic lineage cells tested, PDE4-inhibitor-induced augmentation of GRα transcript levels is specific to B-CLL. B-CLL cells, T-CLL cells, Sezary cells, human peripheral blood mononuclear cells (PBMC), T cells, B cells, neutrophils (PMNs) or monocytes were incubated for four hours in vehicle alone (DMSO) or rolipram (20 μM) as indicated. RNA was isolated, cDNA synthesized, and relative levels of GRα transcript assessed by real-time PCR. The data is representative of at least two samples tested for all cell populations except T-CLL, where only one patient sample was available.

Figure 4: Effect of several structurally distinct PDE4 inhibitors on glucocorticoid-mediated apoptosis and GRα transcript levels. (A) Molecular structures of cilomilast (1), roflumilast (2), and rolipram (3). (B) CLL cells were treated for four hours with vehicle alone (DMSO) or the indicated concentration of PDE4 inhibitor, followed by evaluation of GRα transcript level by real-time PCR. The data are representative of two patients

tested. (C) CLL cells were treated for 48 hours with PDE4 inhibitors alone (-7) or in the presence of increasing dosages of dexamethasone, as indicated, followed by assessment for apoptosis. The PDE4 inhibitors cilomilast (1127), roflumilast (2028) and rolipram were added at the concentrations indicated in the sidebar. The data shown are the mean and SEM of five patients tested. (D) CLL cells were treated for 0, 4, 8, 24 or 48 hours with vehicle alone, rolipram (20 μM), dexamethasone (1 μM), or the combination of rolipram and dexamethasone. For all cell samples treated with drugs for less than 48 hours, the drugs were removed by washing, followed by completion of culture in media alone until 48 hours had elapsed.

Figure 5: Panel A: Treatment of B-CLL cells with the PDE4 inhibitor rolipram alters relative expression of GR transcripts containing exons 1A, 1B, 1C, 8/9α and 8/9β. B-CLL cells from six patients were treated with rolipram (20 μM) for four hours, followed by RNA isolation, cDNA synthesis and real-time PCR for GRα □□□□β transcripts using oligonucleotides that spanned exons 1A3/2 (1A), 1B/2 (1B), 1C/2 (1C), or 8/9α (alpha). The rolipram-induced augmentation of GR transcripts containing exon 1A3 sequence was significantly greater than that of transcripts containing exon 1B (Wilcoxon signed rank test for paired comparisons $p < .01$). A comparable analysis was also carried out for transcripts containing exon 8/9β, □□□□ but here only three patient samples were analyzed. **Panel B: Co-treatment with a PDE4 inhibitor and dexamethasone maintains GRα transcript levels at greater than basal levels despite dexamethasone-induced down-regulation of GRα expression.** B-CLL cells were treated with varying dosages of dexamethasone as indicated for four hours in the presence or absence of rolipram (20 μM), followed by RNA isolation, cDNA synthesis and real-time PCR for GRα using oligonucleotides that spanned exons 8 and 9α. All GR transcript levels were normalized to that observed in DMSO-treated cells.

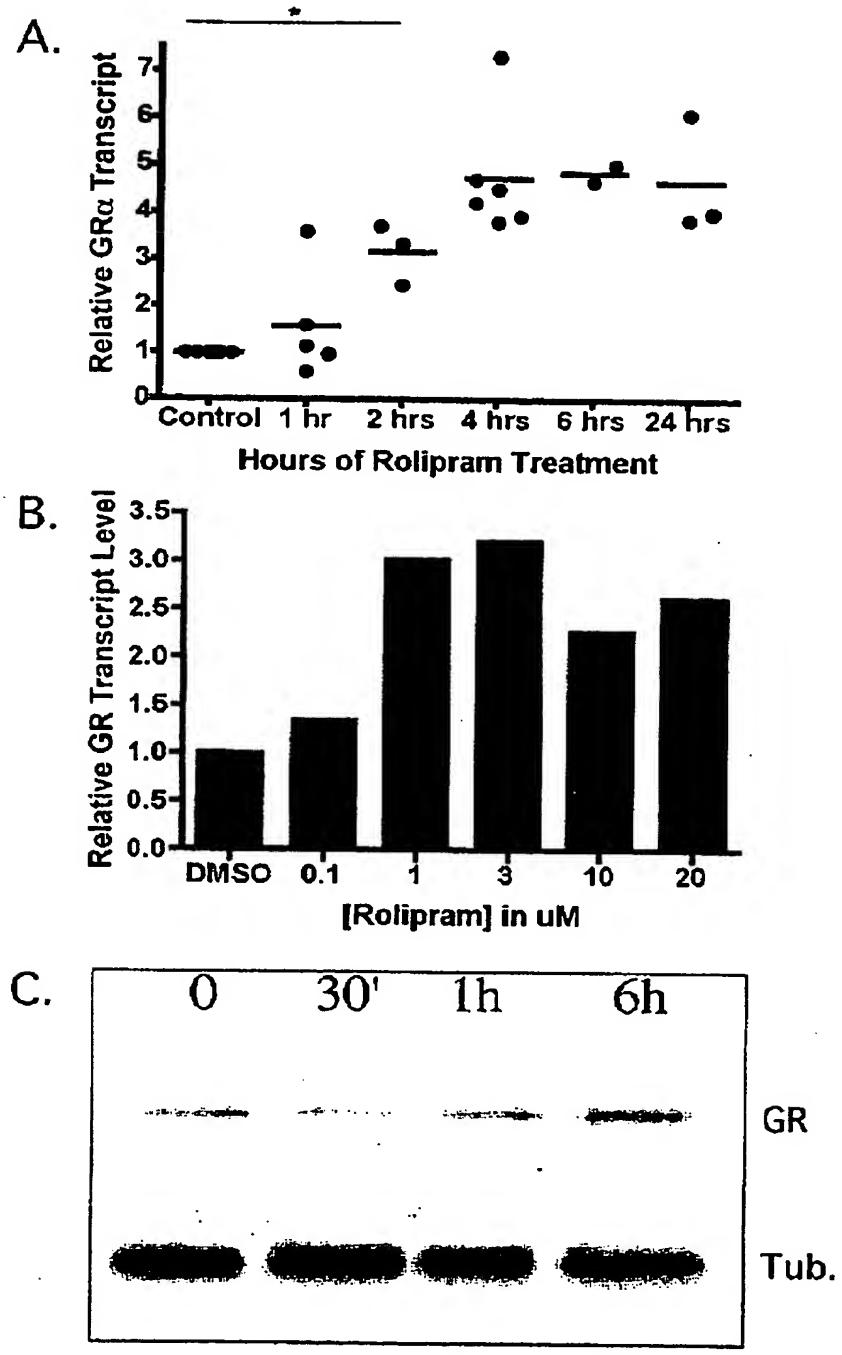
A.
Relative GRα Transcript
0
1
2
3
4
5
6
7
*
Control
1 hr
2 hrs
4 hrs
6 hrs
24 hrs
Hours of Rolipram Treatment
B.
Relative GR Transcript Level
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
DMSO
0.1
1
3
10
20
[Rolipram] in uM
C.
0
30'
1h
6h
GR
Tub.

Figure 1.

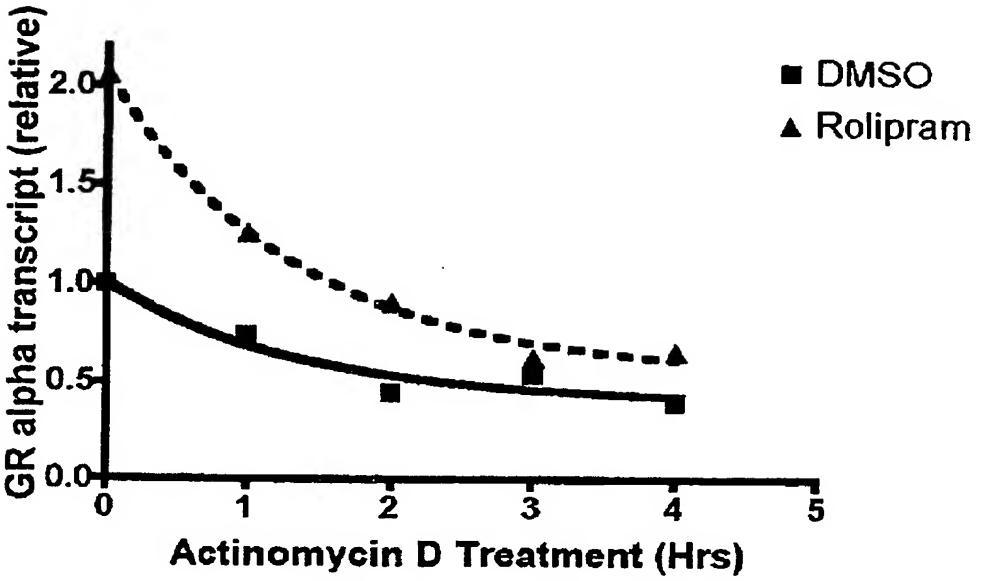
GR alpha transcript (relative)
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Actinomycin D Treatment (Hrs)
DMSO
Rolipram

Figure 2.

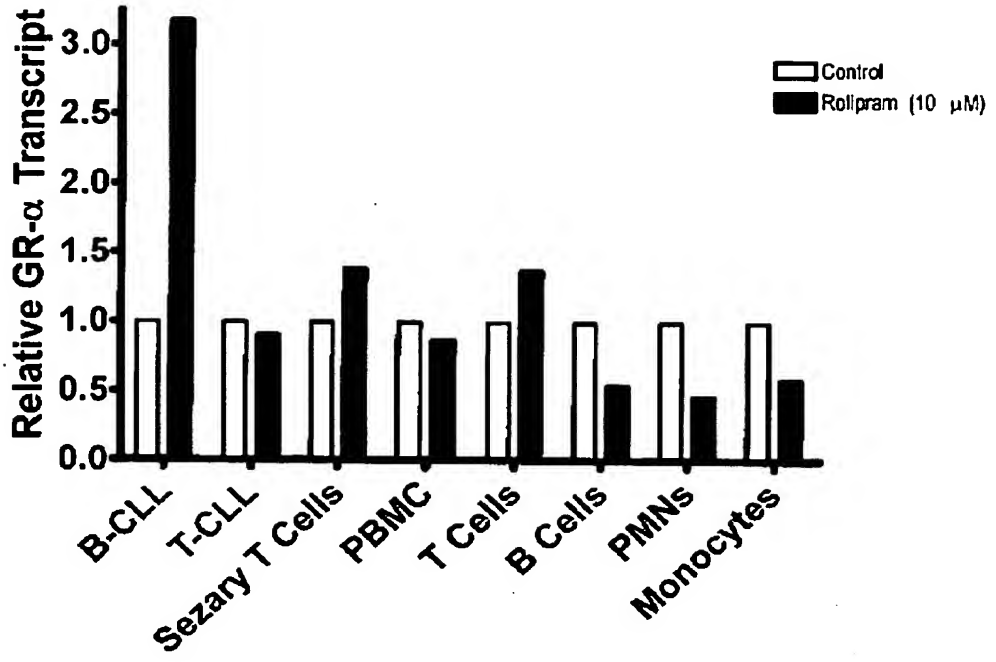
Relative GR-α Transcript
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Rolipram (10 μM)
B-CLL
T-CLL
Sezary T Cells
PBMC
T Cells
B Cells
PMNs
Monocytes

Figure 3.

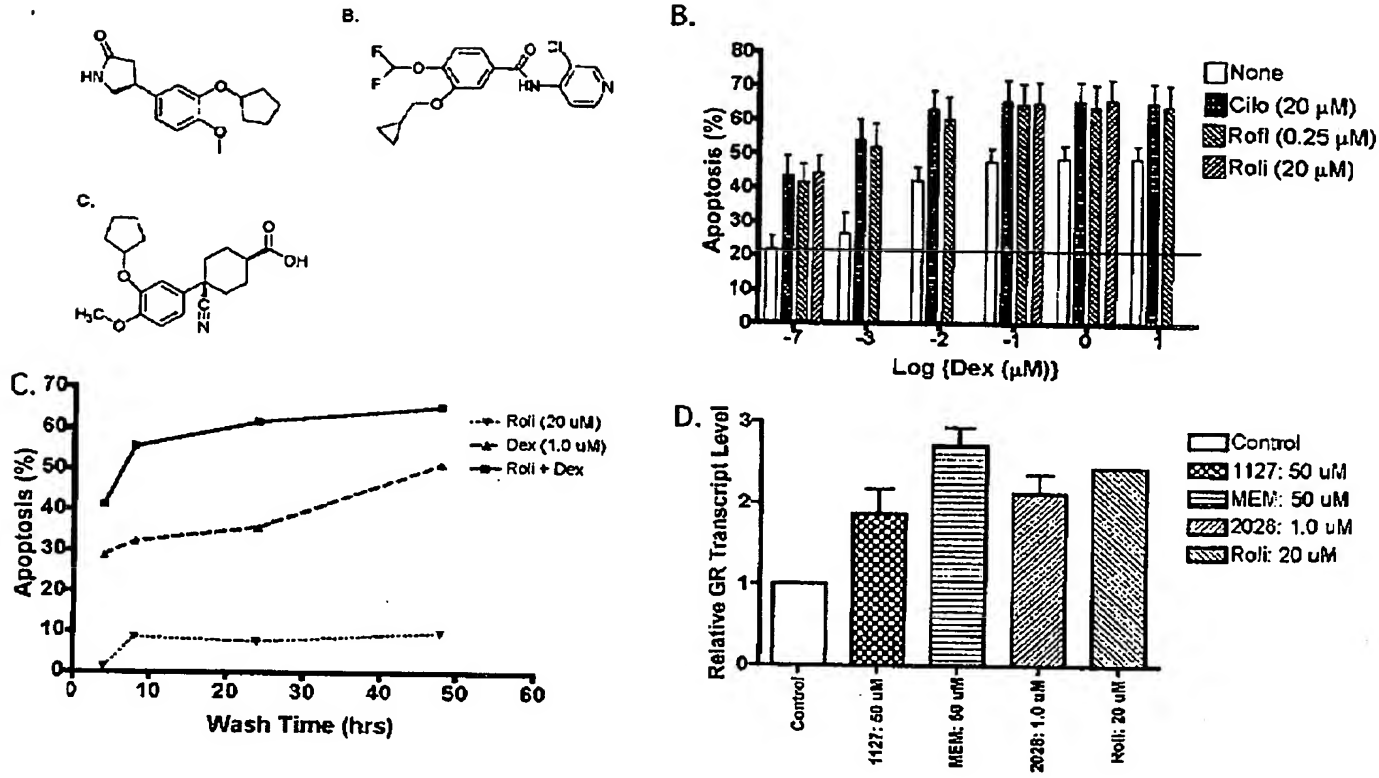
B.
C.
C. 70
Apoptosis (%)
Wash Time (hrs)
Roli (20 uM)
Dex (1.0 uM)
Roli + Dex
B.
Apoptosis (%)
Log (Dex (μM))
None
Cilo (20 μM)
Rofl (0.25 μM)
Roli (20 μM)
D.
Relative GR Transcript Level
Control
1127: 50 uM
MEM: 50 uM
2028: 1.0 uM
Roli: 20 uM

Figure 4

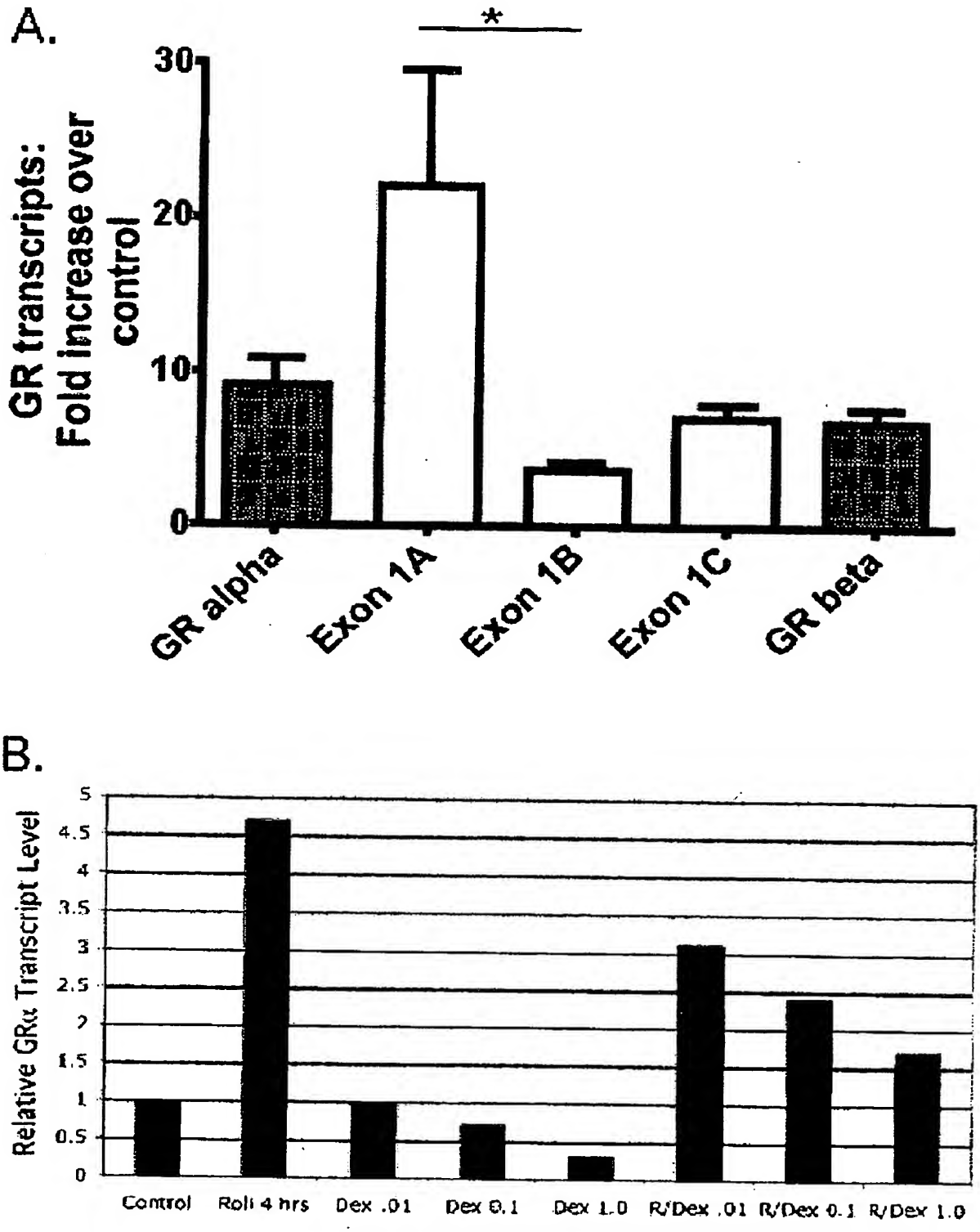
A.
*
GR transcripts: Fold increase over control
30
20
10
0
GR alpha
Exon 1A
Exon 1B
Exon 1C
GR beta
B.
Relative GRα Transcript Level
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Roli 4 hrs
Dex .01
Dex 0.1
Dex 1.0
R/Dex .01
R/Dex 0.1
R/Dex 1.0

Figure 5

References

1. Sawitsky A, Rai KR, Glidewell O, Silver RT. Comparison of daily versus intermittent chlorambucil and prednisone therapy in the treatment of patients with chronic lymphocytic leukemia. Blood. 1977;50:1049-1059.
2. Han T, Ezdinli EZ, Shimaoka K, Desai DV. Chlorambucil vs. combined chlorambucil-corticosteroid therapy in chronic lymphocytic leukemia. Cancer. 1973;31:502-508.
3. Molica S. High-dose dexamethasone in refractory B-cell chronic lymphocytic leukemia patients. Am J Hematol. 1994;47:334.
4. Thornton PD, Hamblin M, Treleaven JG, Matutes E, Lakhani AK, Catovsky D. High dose methyl prednisolone in refractory chronic lymphocytic leukaemia. Leuk Lymphoma. 1999;34:167-170.
5. Moalli PA, Pillay S, Weiner D, Leikin R, Rosen ST. A mechanism of resistance to glucocorticoids in multiple myeloma: transient expression of a truncated glucocorticoid receptor mRNA. Blood. 1992;79:213-222.
6. Irving JA, Minto L, Bailey S, Hall AG. Loss of heterozygosity and somatic mutations of the glucocorticoid receptor gene are rarely found at relapse in pediatric acute lymphoblastic leukemia but may occur in a subpopulation early in the disease course. Cancer Res. 2005;65:9712-9718.
7. de Lange P, Segeren CM, Koper JW, et al. Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. Cancer Res. 2001;61:3937-3941.
8. Rabindran SK, Danielsen M, Stallcup MR. Glucocorticoid-resistant lymphoma cell variants that contain functional glucocorticoid receptors. Mol Cell Biol. 1987;7:4211-4217.
9. Zawydiwski R, Harmon JM, Thompson EB. Glucocorticoid-resistant human acute lymphoblastic leukemic cell line with functional receptor. Cancer Res. 1983;43:3865-3873.
10. Soufi M, Kaiser U, Schneider A, Beato M, Westphal HM. The DNA and steroid binding domains of the glucocorticoid receptor are not altered in mononuclear cells of treated CLL patients. Exp Clin Endocrinol Diabetes. 1995;103:175-183.
11. Gruol DJ, Campbell NF, Bourgeois S. Cyclic AMP-dependent protein kinase promotes glucocorticoid receptor function. J Biol Chem. 1986;261:4909-4914.
12. Gruol DJ, Altschmied J. Synergistic induction of apoptosis with glucocorticoids and 3',5'-cyclic adenosine monophosphate reveals agonist activity by RU 486. Mol Endocrinol. 1993;7:104-113.
13. Kiefer J, Okret S, Jondal M, McConkey DJ. Functional glucocorticoid receptor expression is required for cAMP-mediated apoptosis in a human leukemic T cell line. J Immunol. 1995;155:4525-4528.

14. Doucas V, Shi Y, Miyamoto S, West A, Verma I, Evans RM. Cytoplasmic catalytic subunit of protein kinase A mediates cross-repression by NF-kB and the glucocorticoid receptor. Proc Natl Acad Sci U S A. 2000;97:11893-11898.

15. Gruol DJ, Rajah FM, Bourgeois S. Cyclic AMP-dependent protein kinase modulation of the glucocorticoid-induced cytolytic response in murine T-lymphoma cells. Mol Endocrinol. 1989;3:2119-2127.

16. Dong Y, Aronsson M, Gustafsson JA, Okret S. The mechanism of cAMP-induced glucocorticoid receptor expression. Correlation to cellular glucocorticoid response. J Biol Chem. 1989;264:13679-13683.

17. Penuelas I, Encio IJ, Lopez-Moratalla N, Santiago E. cAMP activates transcription of the human glucocorticoid receptor gene promoter. J Steroid Biochem Mol Biol. 1998;67:89-94.

18. Lerner A, Epstein PM. Cyclic nucleotide phosphodiesterases as targets for treatment of haematological malignancies. Biochem J. 2006;393:21-41.

19. Kim DH, Lerner A. Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. Blood. 1998;92:2484-2494.

20. Moon EY, Lerner A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. Blood. 2003;101:4122-4130.

21. Tiwari S, Felekkis K, Moon EY, Flies A, Sherr DH, Lerner A. Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1, but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. Blood. 2004;103:2661-2667.

22. Tiwari S, Dong H, Kim EJ, Weintraub L, Epstein PM, Lerner A. Type 4 cAMP phosphodiesterase (PDE4) inhibitors augment glucocorticoid-mediated apoptosis in B cell chronic lymphocytic leukemia (B-CLL) in the absence of exogenous adenylyl cyclase stimulation. Biochem Pharmacol. 2005;69:473-483.

23. Fan W-T, Koch CA, de Hoog CL, Fam NP, Moran MF. The exchange factor Ras-GRF2 activates Ras-dependent and Rac-dependent mitogen-activated protein kinase pathways. Curr Biol. 1998;8:935-938.

24. Pedersen KB, Vedeckis WV. Quantification and glucocorticoid regulation of glucocorticoid receptor transcripts in two human leukemic cell lines. Biochemistry. 2003;42:10978-10990.

25. Seldon PM, Meja KK, Giembycz MA. Rolipram, salbutamol and prostaglandin E2 suppress TNFalpha release from human monocytes by activating Type II cAMP-dependent protein kinase. Pulm Pharmacol Ther. 2005;18:277-284.

26. Rennard SI, Schachter N, Strek M, Rickard K, Amit O. Cilomilast for COPD: results of a 6-month, placebo-controlled study of a potent, selective inhibitor of phosphodiesterase 4. Chest. 2006;129:56-66.

27. Timmer W, Leclerc V, Birraux G, et al. The new phosphodiesterase 4 inhibitor roflumilast is efficacious in exercise-induced asthma and leads to suppression of LPS-stimulated TNF-alpha ex vivo. J Clin Pharmacol. 2002;42:297-303.

28. Breslin MB, Geng CD, Vedeckis WV. Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. Mol Endocrinol. 2001;15:1381-1395.

29. Fruchter O, Kino T, Zoumakis E, et al. The human glucocorticoid receptor (GR) isoform {beta} differentially suppresses GR{alpha}-induced transactivation stimulated by synthetic glucocorticoids. J Clin Endocrinol Metab. 2005;90:3505-3509.

30. Koga Y, Matsuzaki A, Suminoe A, Hattori H, Kanemitsu S, Hara T. Differential mRNA expression of glucocorticoid receptor alpha and beta is associated with glucocorticoid sensitivity of acute lymphoblastic leukemia in children. Pediatr Blood Cancer. 2005;45:121-127.

31. Ramdas J, Liu W, Harmon JM. Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Res. 1999;59:1378-1385.

32. Vanderbilt JN, Miesfeld R, Maler BA, Yamamoto KR. Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. Mol Endocrinol. 1987;1:68-74.

33. Reichardt HM, Umland T, Bauer A, Kretz O, Schutz G. Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. Mol Cell Biol. 2000;20:9009-9017.

34. Purton JF, Monk JA, Liddicoat DR, et al. Expression of the glucocorticoid receptor from the 1A promoter correlates with T lymphocyte sensitivity to glucocorticoid-induced cell death. J Immunol. 2004;173:3816-3824.

35. Yudt MR, Cidlowski JA. Molecular identification and characterization of a and b forms of the glucocorticoid receptor. Mol Endocrinol. 2001;15:1093-1103.

36. Lu NZ, Cidlowski JA. Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Mol Cell. 2005;18:331-342.